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Towards the development of a novel zero-discharge willow wastewater treatment system

Fergus Mc Auliffe BSc.



Thesis submitted to the National University of Ireland, Cork, in fulfilment of the requirements for the degree of Doctor of Philosophy

School of Biological, Earth and Environmental Sciences

June 2016

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Prof. Peter Jones
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Declaration

This work has not been previously accepted in substance for any degree and is not being concurrently submitted in candidature for any degree. This thesis is the result of my own independent work/investigation, except where otherwise stated.

_____

Fergus Mc Auliffe

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List of Abbreviations

AMF Arbuscular mycorrhizal fungi
BOD_5 Biological oxygen demand
COD Chemical oxygen demand
DW Dry weight
ECM Ectomycorrhizal fungi
E_o Reference evaporation
E_{pan} Pan evaporation
ET Evapotranspiration
ET_c Actual evapotranspiration
ET_o Reference evapotranspiration
FIA Flow injection analyser
HDPE High-density polyethylene
IA Intercropping advantage
LAI Leaf area index
LOI Loss-on-ignition
LDPE Low-density polyethylene
k_c Crop factor
k_{fs} Field saturated hydraulic conductivity
k_{pan} Pan evaporation factor
odt Oven dry tonnes
OWTS On-site wastewater treatment system
PFT Picket fence thickener
qPCR Quantitative polymerase chain reaction
SBA Stem basal area
SLA Specific leaf area
SRC Short rotation coppice
SVI Stem volume index
T Transpiration
TN Total nitrogen
TP Total phosphorus
TSS Total suspended solids
WWTP Wastewater treatment plant
ZDWS Zero-discharge willow system

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Ní huasal ná íseal ach thuas seal agus thíos seal.

Abstract

Onsite wastewater treatment systems play a vital role in the protection of surface waters, groundwater and drinking water supplies. In areas of low subsoil permeability, where traditional onsite systems are not appropriate, attention has turned to the use of zero-discharge willow wastewater treatment systems (ZDWS). In this thesis, field-scale, meso-scale and lab-scale experimental trials, in combination with field observational studies were carried out, towards the development of a novel ZDWS. Field studies on the use of a polytunnel-covered wetland system found that while evapotranspiration from covered willow systems decreased, the overall efficiency of wastewater removal increased, due to the prevention of incident rainfall from entering the wetland. The development of a novel, polytunnel-covered willow-free wetland system also showed promise, with evaporation rates approaching the evapotranspiration rates achieved by the wetlands containing willows. The use of a winter-active species, *P. fragrans*, to increase winter evapotranspiration was proposed and investigated. Trials found that, due to the inability of the species to tolerate short-duration waterlogging, the distribution of the species in free-draining soils and the low winter evapotranspiration, the use of *P. fragrans* in a zero-discharge system is not effective. In lysimeter trials, willows undergoing an annual coppicing system, necessary for a covered ZDWS, were found to have lower evapotranspiration rates than willows that were not coppiced. Soil clay percentage did not generally affect growth or evapotranspiration for trees undergoing the annual coppicing cycle. Willow crop factors, for use in the sizing of ZDWS in the south of Ireland, were established as part of this trial. Inoculation of the willow trees with a commercial

mixed-species mycorrhizal fungal inoculum increased willow biomass growth. Furthermore, the development of a willow-selected mycorrhizal fungal inoculum led to increased willow shoot biomass and total leaf area compared to treatment with the commercial inoculum. Molecular analysis revealed a change in the relative proportions of the mycorrhizal species, with increased abundances of the disturbance-tolerant species *Glomus mosseae* and *G. intraradices* in the willow-selected inoculum relative to the original commercial inoculum. Overall, despite some modifications and improvements to system efficiency, the achievement of true zero-discharge operation, combined with practical operation, remains difficult in the Irish setting.

Chapter 1 Literature review: domestic wastewater treatment in Ireland and the need for zero-discharge solutions

1.1 Wastewater in the environment

Clean water resources are crucial to maintaining well-functioning rural communities (EPA, 2013). Safe and dependable means of dealing with wastewater are integral to the achievement of clean wastewater resources. A wide range of contaminants are found in domestic wastewater, such as elevated levels of nutrients, organic material, suspended solids, metals, pathogens and parasites (Bitton, 2005). Improperly managed wastewater can lead to environmental pollution and to human health issues, principally gastrointestinal illness (Macler and Merkle, 2000; Dawes and Goonetilleke, 2003; Withers *et al*., 2011). These threats require that wastewater be adequately treated and managed. In urban areas of Ireland a well-developed sewerage network exists, with 95.8% of the urban wastewater load receiving treatment in urban wastewater treatment plants (EPA, 2014a). However, in rural areas, such sewerage infrastructure rarely exists. In the absence of connection to a mains sewage treatment system, wastewater is treated in an on-site wastewater treatment system (OWTS).

1.2 On-site wastewater treatment systems in Ireland

In Ireland, approximately 487,000 OWTS are in operation (Central Statistics Office, 2012) producing 210,000 m^3 of wastewater daily (EPA, 2013). In general, adequate treatment and disposal of domestic wastewater occurs in OWTS which are properly located, constructed, installed and maintained (Siegrist *et al*., 2000; US EPA, 2002; Daly *et al*., 2012). However, underperforming or poorly-sited OWTS can lead to environmental pollution (Harden *et al*., 2008; Withers *et al*., 2011; EPA, 2013), the contamination of private drinking wells (Aldwell *et al*., 1988; Harden *et al*., 2008;

EPA, 2012a) and the contamination of surface waters used for drinking water supplies (Glaberman *et al*., 2002; Lucy *et al*., 2009).

Groundwater supplies approximately 25% of drinking water nationally (EPA, 2012a). There are an estimated 170,000 private wells or boreholes that supply drinking water to 10.6% of the Irish population (EPA, 2015). The contamination of groundwater resources can last for a much longer time than that for surface water due to the longer residence times in groundwater (Gill *et al*., 2006). Thus, the prevention of groundwater pollution from OWTS is crucial to protect human health.

High rates of private well pollution have been reported for Ireland. Almost 25% of private well supplies studied in Co. Cork were found to have faecal contamination (Bacci and Chapman, 2011). In a study of 226 private wells across Ireland, 28.9% tested positive for coliforms, with septic tank setback distance significantly negatively associated with the presence of coliforms (Hynds *et al*., 2012). In the US, over half of waterborne disease outbreaks are due to contaminated drinking water, with septic tanks reported as the most frequent cause of contamination (Keswick and Gerba, 1980; Yates, 1985). Across Ireland, pollution from OWTS or farmyards are regarded as being approximately equal in contribution to the microbial pollution of groundwater (Daly, 2009). There is a widespread lack of awareness among Irish rural homeowners of the threat posed by OWTS with recent research showing that homeowners with private wells showed the lowest recognition of OWTS as a potential source of aquatic contamination, despite being the group at greatest risk (Naughton and Hynds, 2014).

OWTS can negatively affect the quality of surface water bodies as a result of incomplete treatment, overland flow to streams or direct discharge to streams (Edwards and Withers, 2008; McCarthy *et al*., 2009). In Ireland, OWTS contribute 7% of the phosphorus loading and 3% of the total nitrate to surface waters (ESB International, 2008). Phosphorus arising from OWTS is the pollutant of most concern for surface water contamination (EPA, 2012b). OWTS can increase in-stream nutrient concentrations especially during the drier summer months at very low flows (Douglas *et al*., 2007; Macintosh *et al*., 2011; Withers *et al*., 2012).

The spread of once-off housing and associated OWTS across Ireland has led to the rural Irish landscape being regarded as an intermediary between the traditional agricultural (diffuse-source) and traditional urban (point-source) landscapes for sources of wastewater pollution (Arnscheidt *et al*., 2007; Withers and Jarvie, 2008). While the overall national risk from OWTS is lower than that from agricultural activities and urban WWTPs, there are areas of Ireland, such as parts of counties Wexford, Limerick and Monaghan, that are at a local risk from OWTS due to the prevailing ground conditions and the density of OWTS (Sweeney and Keegan, 2012). Due to the threats posed by OWTS to human health and the environment, extensive historical and current legislation govern the use of these systems in Ireland. The legislation and guidelines are summarised below.

1.2.1 Historical and current legislation for OWTS

In 1975 the Irish Institute for Industrial Research and Standards published the guidelines *"Recommendation for Septic Tank Drainage Systems Suitable for Single Houses"* (SR6:1975) which outlined best practice for septic tank systems in Ireland.

In 1991, the National Standards Authority of Ireland published the guidelines "*Septic Tank Systems: Recommendations for Domestic Effluent Treatment and Disposal from a Single House"*. These recommended that a site suitability assessment be carried out and gave recommendations as to how percolation areas should be constructed. In 2000, the EPA published the guideline manual "*Wastewater Treatment Manual: Treatment Systems for Single Houses"* which further defined the site assessment steps to be taken and acceptable percolation limits.

The most recent guidelines for Ireland were published in 2009 as the "*Code of Practice: Wastewater Treatment and Disposal Systems serving Single Houses (p.e. ≤ 10)"* (EPA, 2009). These guidelines outline the entire site assessment process and give recommendations to the range of primary, secondary and tertiary systems that can be installed. Primary wastewater treatment is the most basic form of treatment and mainly involves the settling out of solids. Secondary treatment reduces the organic loading, nutrient loading and microorganism numbers in primary treated wastewater. Tertiary treatment occurs when particular pollutants, such as N and P, require further reduction.

When installing an OWTS a site suitability assessment must be undertaken. The aim of the site suitability assessment is to protect groundwater, to assess the vulnerability of local groundwater resources and to identify any receptors, e.g., surface water and nearby wells that are potentially at risk (EPA, 2009). The site-suitability assessment involves a desk study, followed by a visual assessment. Trial holes are excavated followed by the T-test. This is a falling head percolation test

whereby the time taken for a column of water to drop a distance of 100 mm in a 300 x 300 x 400 mm hole is recorded. Recommendations are then made on the most suitable wastewater treatment system and disposal route to install on the site.

The 2009 guidelines only apply to new systems and not to systems that pre-date the publication of the Code of Practice. In the case of new houses, under Article 22(2)(c) of the Planning and Development Regulations 2006 (S.I. No 685 of 2006), planning permission applications for sites on which wastewater disposal will be on site must include details of the proposed wastewater treatment system and the suitability of the system for that site. All new OWTS must meet the requirements of the EN 12566 series of standards (European Committee for Standardisation).

The typical pollutant concentration ranges for raw domestic wastewater and the minimum performance standards specified under the Code of Practice are shown in Table 1.1. Minimum performance standards are prescribed for Biological Oxygen Demand (BOD_5), Total Suspended Solids (TSS) and ammonium (NH_4-N). In nutrient-sensitive locations minimum performance standards for Total Nitrogen (TN) and Total Phosphorus (TP) are also given.

Table 1.1 Pollutant concentration values for raw domestic wastewater and minimum performance standards following treatment by OWTS (EPA, 2009).

Pollutant	Raw domestic wastewater (mg L^{-1})	Minimum performance standard in effluent (mg L^{-1})
BOD_5	150-500	20
TSS	200-700	30
NH_4-N	22-80	20
TN	50	5[a]
TP	5-20	2[a]
COD	300-1000	N/A
Total coliforms	10^6-10^9 MPN[b] 100 mL^{-1}	N/A

[a] Only for nutrient-sensitive locations. [b]Most Probable Number.

In 2009, the European Court of Justice ruled that Ireland had failed to bring in necessary legislation regarding OWTS to comply with Articles 4 and 8 of the Council Directive 75/442/EEC, "The Waste Directive" (Case C-188/08). In response, the Irish government introduced the Water Services (Amendment) Act 2012, which led to the EPA publishing the National Inspection Plan 2013. Under the Act, homeowners have a duty of care to operate and maintain their OWTS to ensure that domestic wastewater treatment systems do not pose a risk to human health or the environment.

The National Inspection Plan and the Code of Practice are designed to help Ireland to meet its targets under the Water Framework Directive (Directive 200/60/EC)(European Commission, 2000). The Directive aims to protect all high status waters, prevent the further deterioration in quality of all waters and return degraded surface waters and groundwater to "good status" by December 2015. Tackling pollution from OWTS is regarded as a major challenge in ensuring that Ireland meets the requirements of the WFD (EPA, 2012a).

1.2.2 Assessment and alterations to the septic tank system

Under the National Inspection Plan 2013, OWTS are inspected by trained Local Authority inspectors to ensure that treatment systems are fit for purpose and do not pose a threat to the environment or to human health. The inspection includes an assessment of any leaks from the system, whether the septic tank has been de-sludged, whether surface ponding occurs and whether the system is being properly operated and maintained. During the first eight months of the operation of the inspection plan (July 2013 – February 2014) a total of 423 inspections were carried out, with a compliance rate of 47% (EPA, 2014b). Evidence of surface ponding was found to occur in 13% of systems inspected. Of the total number of systems inspected, 33% were deemed by the inspectors to pose a threat to human health or the environment (EPA, 2014b).

In the case of non-compliant systems, the local authority issues an advisory notice. This notice is closed once improvement measures are complete, which usually occurs within three to six months. Depending on the nature of the improvements required, considerable expense can occur. Grants up to a maximum value of €4,000 are available under the Domestic Wastewater Treatment Systems (Financial Assistance) Regulations (S.I. No. 222 of 2013).

Many options for retro-fitting improvements to septic tanks exist, such as ECO-FIT® and Cloudburst Retro®. These technologies focus on improving the quality of the septic tank effluent using aeration or activated sludge, before it passes into the percolation area. However, in cases where the soil/subsoil beneath the percolation area cannot take the hydraulic loading and surface ponding occurs, these retro-fit

improvements are not suitable as the fundamental issue of subsoil drainage is unchanged.

1.3 On-site treatment options for domestic wastewater

OWTS can be divided into two broad categories: septic tank systems and mechanical aeration systems. A traditional septic tank system consists of a septic tank followed by a conventional percolation area (EPA, 2009). As an alternative to the conventional percolation area a soil/sand/peat filter or a constructed wetland can be installed, followed by a polishing filter. A polishing filter is a sand filter for wastewater infiltration to the subsoil and is used to remove microorganisms and phosphorus in otherwise high quality wastewater (EPA, 2009). Mechanical aeration systems include Biological Aerated Filter systems, Rotating Biological Contactor systems, Membrane Bioreactor systems and Activated Sludge systems and are followed by a polishing filter. For a review of the performance of mechanical systems in Ireland see Dubber and Gill (2012).

The septic tank and percolation area system is the most common OWTS in Ireland, accounting for 437,000 (89.7%) of households with on-site treatment (Central Statistics Office, 2012). These systems are of particular importance to Ireland due to the dispersed rural population. For perspective, in Ireland the ratio of septic tank systems to head of the population is 1:11, while in England and Wales the ratio is 1:65 (Smyth *et al*., 2012). Due to the particular importance of septic tanks and percolation areas for Ireland, the present review will examine conventional septic tank systems in detail, in particular septic tank and percolation areas, and septic tanks followed by constructed wetlands.

1.3.1 Septic tanks

Fundamentally, septic tanks are enclosed containers that receive wastewater, collect and degrade solids through passive anaerobic digestion, and discharge the treated effluent (Bounds, 1997). The septic tank must be situated at least 7 m from the house (EPA, 2009). Both blackwater (liquid from toilets) and greywater (liquid from washing machines, dishwashers etc.) flow from the house to the septic tank. Septic tank systems are a sustainable means for wastewater treatment due to low energy and chemical requirements (Muga and Mihelcic, 2008).

A popular misconception exists among the general public that septic tanks treat wastewater to a standard where no further treatment is required (Gill, 2011). While septic tanks can accomplish more than 45% of the total wastewater treatment (Bounds, 1997), the purpose of the septic tank is to provide primary treatment by reducing the amounts of solids in the wastewater through sedimentation and anaerobic digestion (Bouma, 1979; Beal *et al*., 2005). Depending on performance, a septic tank can remove 15-80% of the BOD and 50-80% of the solids (Bouma, 1979; Gill *et al*., 2004; Bitton, 2005). Desludging of the accumulated solids should occur at a frequency appropriate to the tank volume and the number of persons resident in the house (Domestic Wastewater Treatment Systems Regulations, 2012). Septic tank effluent contains large numbers of bacteria, virus particles and parasites. There are approximately 1 million *E. coli* bacteria and 10 million faecal coliforms in a litre of septic tank effluent (EPA, 2009). Virus particle concentrations in septic tank effluent are 10^7 – 10^{10}-fold higher during times of infection in the household

(Canter and Knox, 1985). The drinking water standard for *E. coli* and enterococci is zero per 100 ml (EPA, 2010).

For nutrient removal, within the anaerobic environment of the septic tank, little reduction in overall nitrogen and phosphorus occurs (Gill *et al*., 2004). Nitrogen in septic tank effluent is mainly in the form of organic nitrogen and ammonium (Bouma, 1979) with typical ammonium concentrations of 44-55 mg L^{-1} for Irish septic tanks (Gray, 1995; Gill *et al*., 2007). The nitrate concentrations in septic tank effluent are very low (usually zero) due to the lack of aerobic conditions (Gill *et al*., 2004). Much of the organic phosphorus is converted to soluble orthophosphate (Beal *et al*., 2005). Limited TP removal occurs with typical influent TP of 25 mg L^{-1} and typical effluent TP of 15 mg L^{-1} (Gill *et al*., 2004). Effluent orthophosphate levels have been recorded as 8-14 mg L^{-1} (Gill *et al*., 2007). Thus, despite undergoing treatment within the septic tank itself, effluent from septic tanks is still highly polluting and requires further treatment.

1.3.2 Percolation area

OWTS have historically relied on soil for secondary wastewater treatment and disposal (McKinley and Siegrist, 2011). A percolation area, including the soil and subsoil, is considered to be the most important part of the wastewater treatment process (Carroll *et al*., 2006; Gill *et al*., 2007) and is effectively the "last line of defence" to prevent pollution of groundwater (Dawes and Goonetilleke, 2003). The principal aquifers in Ireland occur in fissured bedrock which offer little pollutant attenuation, and thus the soil and subsoil act like a protective cap to prevent groundwater contamination (Swartz *et al*., 2003).

A percolation area consists of a distribution device to spread the septic tank effluent evenly along a series of subsurface trenches that are laid down in gravel beds. Wastewater moves under gravity flow along perforated distribution pipes within the trenches before passing through the gravel and into the soil/subsoil (EPA, 2009). A minimum depth of 1.2 m of soil/subsoil must lie between the base of the percolation trenches and the water table or bedrock. Percolation areas can also be raised where an inadequate depth to water table or depth to bedrock exists.

The maximum length of any individual trench is 18 m, with a length of trench of 18 m person^{-1} advised. Under the Code of Practice, restrictions on the siting of a percolation area apply. A minimum distance of 10 m must lie between the percolation area and the residence. A percolation area must be a minimum of 10 m from the nearest watercourse, a minimum of 50 m from the nearest lake and a minimum of 60 m from the nearest public water supply. Adequate separation between a percolation area and a private well must exist, though this is dependent on the result of the T-test, the type and depth of soil/subsoil and the location of the well with respect to the slope. A minimum of 15 m separation must lie between a percolation area and an up-gradient well. For a down-gradient well there must be a minimum of 30-60 m depending on the site-specific factors outlined above (EPA, 2009).

Secondary treatment occurs within a percolation area through a number of biogeochemical and physical processes in the biomat and subsoil (Beal *et al*., 2005). The biomat forms along the wetted sides of a percolation area at the interface between the soil and gravel. Biomat formation occurs due to clogging caused by

suspended solids from the wastewater becoming trapped at the soil/gravel interface and the build-up of humic-like substances which form a “glue” that retains water and solid materials (Siegrist *et al*., 2012). The majority of the biological breakdown of the wastewater occurs in the biomat zone (Gill *et al*., 2004; McKinley and Siegrist, 2011). With time, the hydraulic conductivity of the biomat decreases, leading to a saturated zone above the biomat but an unsaturated zone beneath it (Beal *et al*., 2005; McKinley and Siegrist, 2011). After a few months of operation of a percolation area, an infiltration steady state is reached, known as the long-term acceptance rate. This is the rate at which the soil can continue to accept pre-treated effluent without hydraulic failure occurring (Siegrist *et al*., 2000; Beal *et al*., 2005; Gill *et al*., 2007).

Across the biomat and percolation area zone, removal rates of BOD, COD and TSS of more than 90% are common by physical straining through soil pores and through microbial degradation of the organic fraction (Siegrist *et al*., 2000; Gray, 2004; Siegrist *et al*., 2012). A percolation area is capable of >99% removal of bacteria and viruses (van Cuyk *et al*., 2001). Pathogenic bacteria are removed through straining or adsorption (the removal from soil solution and accumulation at the soil-liquid interface)(Canter and Knox, 1985) and subsequent inactivation (Stevik *et al*., 2004). Virus particles become adsorbed and inactivated on soil grain surfaces (Reddy *et al*., 1981).

The TN removal performance of septic tank systems relies primarily on the soil attenuation capability of a percolation area (EPA, 2009). Nitrification of the ammonium in the septic tank effluent occurs in the unsaturated aerobic zones of

the subsoil (Walker *et al*., 1973; Gill *et al*., 2006). TN removal is achieved by bacterial denitrification in the anaerobic saturated zones within the subsoil as the organic loading of the septic tank effluent supports the multiplication of denitrifying bacteria (Gill *et al*., 2006). The main processes for phosphorus removal are precipitation, adsorption and complexation (Siegrist *et al*., 2000). Phosphorus removal is highly dependent on the nature of the subsoil, in particular on the amount of clay particles in the subsoil (Gill *et al*., 2009). Aluminium, iron and manganese oxides are important for phosphorus adsorption in acidic soils, while the presence of calcium for precipitation is more important in alkaline soils (Siegrist *et al*., 2000).

1.3.3 The importance of adequate percolation to the performance of a traditional septic tank and percolation area system

The Code of Practice currently advises a minimum depth of 1.2 m of unsaturated subsoil below the invert of a percolation trench which receives septic tank effluent (EPA, 2009). The presence of an adequate depth of unsaturated subsoil beneath the biomat is crucial to treatment performance (Beal *et al*., 2005). In the unsaturated zone there is prolonged contact between the wastewater and the soil medium as the negative soil water potential forces effluent to flow through the water that adheres to the soil surface particles (US EPA, 2002). The macropores of the unsaturated zone are generally filled with air leading to aerobic degradation of effluent by the microorganisms that grow on the surface of the soil particles (Beal *et al*., 2005).

The majority of the effluent treatment occurs in the first 0.6 m below the percolation pipes. Gill *et al.* (2007) found that septic tank effluent, after travelling through a depth of 0.6 m of unsaturated subsoil, was of a quality equivalent to septic tank effluent that had received secondary treatment in a peat biofilter (Puraflo®). At a depth of 1.0 m below the percolation pipes, the effluent quality is acceptable for discharge to groundwater (Gill *et al*., 2005).

One of the key questions that the site characterisation step of the Code of Practice aims to answer is, *"Is the site able to dispose of the wastewater volumes?"* Under conditions of poor subsoil drainage, the issue of wastewater disposal becomes a problem. In areas where poor subsoil permeability exists, such that the subsoil cannot accept the hydraulic loading of the effluent, surface ponding can occur (with associated health risks) as well as a risk of overland flow of effluent and surface water pollution (McCarthy *et al*., 2009; Gill, 2011; Dubber *et al*., 2014). In low permeability sites, wastewater can be forced to flow upwards or laterally through distinct pathways such as sand lenses, leading to inadequate treatment (Keegan *et al*., 2014). Furthermore, saturated subsoil can lead to poorly treated wastewater reaching groundwater due to shortened hydraulic retention times and reduced aerobic conditions (Beal *et al*., 2005).

Permeability is most affected by soil texture, principally the percentage of clay particles present and how the grains are packed together (Meehan and Lee, 2012). During the on-site assessment, the suitability of the subsoil to hydraulically transmit effluent away from the treatment system is assessed through the T-test. In a study of 198 T-tests, sand-dominated subsoils had an average T-value of 14.2 (minutes

per 100 mm drop), silt-dominated subsoils had an average T-test value of 21, and silt/clay subsoils averaged 32.1. The T-test is failed when a value of > 90 is returned and the results for clay subsoils were all over 100 (Meehan and Lee, 2012). High T-test results are primarily because low permeability subsoils do not have sufficient pore space to allow the wastewater to run through and because they contain higher proportions of silt and clay particles (Swartz *et al*., 2003; Lee *et al*., 2008).

The particle size distribution analysis of low permeability sites shows that these sites generally have greater than 36% fines (silt + clay) and tend to have at least 13% clay (Swartz *et al*., 2003). Glacial till/boulder clay is the most common subsoil across Ireland, covering 43% of the country at the subsurface level (Meehan and Lee, 2012). Glacial tills are generally regarded as being of moderate to low permeability due to a high proportion of silt or clay (Misstear *et al*., 2009).

Susceptibility matrices have been developed for Ireland using the "Source-Pathway-Receptor" model which categorises the country into areas of "low", "medium", "high" and "very high" susceptibility to inadequate percolation and attenuation of nitrate, molybdate-reactive phosphate and microbial pathogens (Daly *et al*., 2012). In areas classified as "very high" (26% of the land surface area), there is an 80% likelihood of finding inadequate percolation, whereas, in sites classified as "low" susceptibility (25% of the land surface area), there is less than a 5% chance of finding inadequate percolation.

A national map, at 1:40,000 scale, has been created showing the likelihood of inadequate percolation due to poor subsoil/bedrock permeability (Figure 1.1).

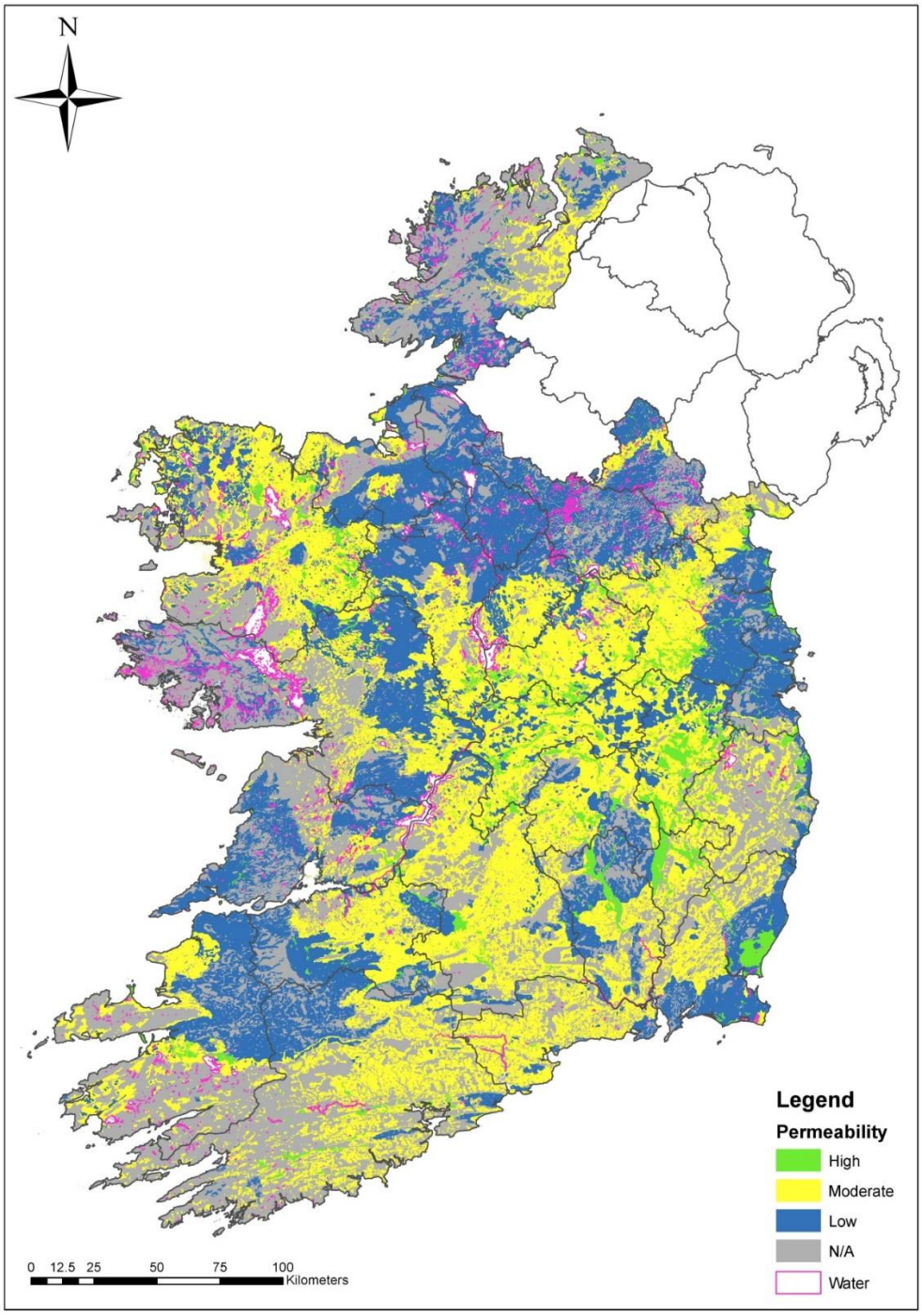


Figure 1.1 Subsoil permeability map of Ireland showing subsoil classes divided into areas of high, medium, low, N/A and water bodies. (Image: Monica Lee, Geological Survey of Ireland).

Nationally, 39% of the land area of Ireland experiences inadequate drainage conditions for all or part of the year (EPA, 2012b). In sites deemed unsuitable for

the discharge of effluent to ground it is usually due to poorly draining soil/subsoil, and/or poorly permeable bedrock (EPA, 2009). It is generally accepted that discharge to ground in clay-dominated subsoils will not work effectively (Meehan and Lee, 2012).

Site improvement works, such as the importing of higher permeability soils, incur a high cost and in many cases are unlikely to work. Under the Code of Practice (EPA, 2009) the only remaining option is effluent disposal to surface water which requires a Water Pollution Licence. In general, local authorities do not grant these licences for single houses (EPA, 2009). With such a high proportion of Ireland experiencing inadequate percolation, a solution is needed to overcome this challenge

1.4 Constructed wetlands

An alternative to a traditional percolation area is the constructed wetland (CW) or artificial reedbed. These are engineered systems which use plant vegetation, soil and microbial processes in the treatment of wastewaters (Vymazal, 2010). Developed in Germany in the 1950s, CW gained popularity in Ireland during the 1990s, with at least 140 CWs present in Ireland as of 2005 (Babatunde *et al.*, 2008). CWs are regarded as a sustainable and low-cost method for domestic wastewater treatment (O'Luanaigh *et al.*, 2010). Under the Code of Practice (EPA, 2009), CWs can be used as either secondary or tertiary wastewater treatment systems. CWs must be sealed with an impermeable clay or geotextile liner and followed by a soil/sand polishing filter.

CWs can be classified according to the direction of wastewater flow (vertical or horizontal), the presence or absence of surface water (surface or sub-surface flow)

or by the type of vegetation (emergent, floating, submerged etc.) (Vymazal, 2008). For the purpose of this review, CWs have been classified into two types: surface flow (SF), and subsurface flow (SSF – of which there are two sub-types: vertical flow (VFSSF) and horizontal flow (HFSSF)).

SF are soil-based wetlands with horizontal flow and contain areas of open water with emergent, submerged and floating plant vegetation (Kadlec and Wallace, 2009). Under the Code of Practice (EPA, 2009) the area demand for SF is 20 m^2 $p.e.^{-1}$ (population equivalent – 60 g BOD $person^{-1}$ day^{-1}) for secondary treatment and 10 m^2 $p.e.^{-1}$ for tertiary treatment. In Ireland, SF are typically used to treat agricultural or domestic wastewater (Babatunde *et al*., 2008). In general, SF have poorer treatment levels on a unit area basis compared to SSF systems and tend to be much larger (EPA, 2009). BOD and TSS removal rates can be greater than 85% (Healy and O'Flynn, 2011), while removal rates of 37 - 77% for TN and 13 - 64% for TP have been reported for Ireland (Healy and Cawley, 2002; Healy and O'Flynn, 2011). Most of the P removal occurs through adsorption to the soil with removal through harvest of wetland vegetation being almost insignificant (Healy and Cawley, 2002).

There are two sub-types of SSF wetlands: horizontal flow (HF) and vertical flow (VF). HFSSF systems (commonly known as "reed beds") move wastewater from the inlet through gravel to an outlet pipe without the wastewater breaching the surface of the medium (Vymazal, 2008). Macrophytes such as *Phragmites australis* Trin ex Steudel (common reed)*, Iris pseudacorus* L. (yellow flag iris) and *Typha latifolia* L. (common cattail) are planted into the gravel medium. HFSSF systems have high

removal rates for organics in both the aerobic and anaerobic zones of the wetland, and for TSS due to physical straining and settling in the wetland media (Vymazal and Kröpfelová, 2008). Low rates of nitrogen removal are common, due to poor oxygenation of the substrate leading to incomplete nitrification (Vymazal and Kröpfelová, 2008). Nitrogen removal rates of 29% for secondary effluent and 41% for tertiary effluent have been reported in the Irish setting (O'Luanaigh *et al*., 2010). Phosphorus removal is also low with 45% removal for secondary wastewater and 22% for tertiary wastewater (O'Luanaigh *et al*., 2010), mainly due to the low quantities of Fe, Al and Ca in the crushed gravel used as substrate (Vymazal, 2008). Removal of phosphorus through plant harvesting is very low (<10% of total inflow) (O'Luanaigh *et al*., 2010). HFSSF are sized at 5 m^2 $p.e.^{-1}$ when used for secondary treatment and 1 m^2 $p.e.^{-1}$ when used for tertiary treatment (EPA, 2009).

In VFSSF wetlands, wastewater is dosed intermittently along the surface of the wetland containing macrophytes, where it drains vertically through graded gravel/sand media (EPA, 2009). Unlike HFSSF, in VFSSF the wastewater drains vertically, and, in between the intermittent pulses of applied wastewater, air diffuses into the medium pores, facilitating good aeration (Vymazal, 2010). VFSSF systems provide good removal of solids and organics (Vymazal *et al*., 1998). VFSSF wetlands are better suited to the treatment of wastewater high in ammonia due to their enhanced ability to nitrify ammonia under aerobic conditions, but their ability to denitrify is limited (Luederitz *et al*., 2001; Vymazal, 2008; Kadlec and Wallace, 2009). Phosphorus removal in VFSSF wetlands is usually low (20-30%) unless chemical precipitation with aluminium polychloride is carried out in a

sedimentation tank preceding the VFSSF (Brix and Arias, 2005a). A special substrate medium, such as alum sludge, can also be used in unplanted VFSSF systems to remove P (Babatunde and Zhao, 2009). Gravel VFSSF systems are sized at 1.5 – 3 m^2 $p.e.^{-1}$ for secondary treatment and 1 m^2 $p.e.^{-1}$ for tertiary treatment. Sand VFSSF wetlands are sized at 5 – 6 m^2 $p.e.^{-1}$ for secondary treatment and 3 m^2 $p.e.^{-1}$ for tertiary effluent (EPA, 2009).

Other types of CWs are in operation such as hybrid systems and Integrated Constructed Wetlands (ICWs). Hybrid systems usually consist of a combination of VFSSF, HFSSF and SF wetlands in a staged manner and are particularly useful for TN removal due to recirculation of nitrified effluent (Vymazal *et al*., 1998; EPA, 2009). ICWs are SF wetlands designed to "fit" into the landscape and to provide amenity (Harrington *et al*., 2010). ICWs have been used to treat domestic wastewater in Ireland, but are typically attached to farms due to the large area requirement (Scholz *et al*., 2007; Dzakpasu *et al*., 2011).

1.5 Constructed wetlands and evapotranspiration systems

A high-performing OWTS can result in successful wastewater attenuation, but it can never overcome the problems posed by fundamentally poorly draining subsoils (EPA, 2012b). Evapotranspiration (ET) systems seek to solve this problem. ET systems are treatment systems whereby wastewater is disposed of to the atmosphere through a combination of soil evaporation and transpiration (US EPA, 2000). Wetland plants have inherently low water use efficiencies as they are adapted to living in environments where soil moisture is not limiting (Headley *et al*., 2012; Randerson *et al*., 2012). CWs planted with *P. australis* have been used in

warmer climates to remove water due to the high transpiration rate (> 4 mm day^{-1})(Bialowiec *et al*., 2011; Headley *et al*., 2012; Białowiec *et al*., 2014). Crop factors (the ratio of actual evapotranspiration to reference evapotranspiration) can be used to study the evapotranspiration rates of plant species under various weather conditions (Allen *et al*., 1998). In an Italian study, a *P. australis*-planted SSF had maximum crop factor values of 6.3 – 8.2 during the summer (Borin *et al*., 2011). However, in colder climates such as the UK and Ireland, evapotranspiration rates are low and a crop factor of < 1.4 is generally used for *P. australis* CWs (Peacock and Hess, 2004; O'Luanaigh, 2007; O'Luanaigh *et al*., 2010), although this can rise to > 2 in late summer (Fermor *et al*., 2001). In the Irish climate, reed beds containing *P. australis*, *T. latifolia and I. pseudacorus* do not significantly reduce the wastewater volume passing through the CW (O'Luanaigh and Gill, 2012). Thus, where poor soil/subsoil exists beneath the subsequent polishing filter, surface ponding can still occur.

In arid and semi-arid climates (where evaporation exceeds precipitation by at least 600 mm per annum) below-ground dispersal of septic tank effluent into a sand-based evaporation bed can be used (US EPA, 2000). Effluent rises to the surface through capillary action where it is evaporated. Subsurface effluent is also transpired by the growing vegetation. Mechanical aeration systems have been used as evapotranspiration systems (US EPA, 2000). However, due to the Irish climatic conditions where rainfall far exceeds evapotranspiration, these systems are unlikely to work. Thus, in areas of Ireland where poor subsoil permeability exists, an OWTS

that can remove all the incoming effluent and precipitation through evapotranspiration is needed.

As a potential solution for existing sites experiencing poor drainage conditions, or new sites seeking planning permission where a T value > 90 exists, attention has turned to the use of zero-discharge willow wastewater treatment systems (ZDWS)(Gill *et al.*, 2016). ZDWS are regarded as a novel constructed wetland system for the treatment of domestic wastewater (Gregersen and Brix, 2001; Brix and Arias, 2005b) and can potentially be used to combine both the traditional CW and polishing filter in one step. This treatment system is reviewed in Chapter 2.

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Chapter 2 Literature review: Improving the efficiency of zero-discharge willow systems in Ireland

2.1 Willows for wastewater treatment

Willows have been used extensively in the treatment of various types of wastewaters, including domestic effluent, landfill leachate, dairy parlour washings, sewage sludge, polluted groundwater and human urine (Roygard, 1999; Gregersen and Brix, 2001; Larsson *et al*., 2003; Duggan, 2005; Aronsson *et al*., 2010; Dimitriou and Aronsson, 2011; Białowiec *et al*., 2012; Guidi Nissim *et al*., 2014). Willows are an ideal species to use in the treatment of wastewater for a variety of reasons, which include: tolerance of flooded conditions, increase in biomass production in response to effluent application, high rates of nutrient removal and high transpiration rates (Table 2.1). Willow short rotation coppice (SRC) has a very high evapotranspiration rate, which exceeds that of other agricultural crops or tree species grown in the UK (Hall *et al*., 1998). The high evapotranspiration rate of willows allows their use in scenarios where wastewater removal, rather than treatment, is required (Białowiec *et al*., 2007a). Northern European domestic wastewater, in terms of nutrients, is very close to the nutrient levels required by willows (Perttu and Kowalik, 1997). Thus, willows are ideally suited to the treatment and disposal of domestic wastewater.

Table 2.1 The suitability of willows for wastewater treatment.

Characteristic	Species	Region	Reference
Tolerance of flooded conditions	*S. viminalis* L.	UK	Jackson and Attwood, 1996
	Fifteen willow species	USA	Kuzovkina *et al.,* 2004
High transpiration rate during the growing season	*S. viminalis*	Sweden	Lindroth and Båth, 1999
	S. alba L.	Italy	Guidi, 2008
High nutrient removal through biomass harvesting	*S. alba, S. alba* var. sanguinea, *Salix x rubra*, *S. purpurea* L., *S. dasyclados* Wimm.	USA	Adegbidi *et al.,* 2001
	S. viminalis	Ireland	Curneen and Gill, 2014
High nutrient removal as willow filter	*S. viminalis*	Sweden	Aronsson and Bergström, 2001
	S. viminalis, S. dasyclados	Sweden	Aronsson *et al.*, 2000
	S. miyabeana Seemen.	Canada	Guidi Nissim *et al*., 2015
High yield in response to nutrient application	*S. viminalis*	UK	Sugiura *et al.*, 2008
	S. viminalis	Ireland	Curneen and Gill, 2014
Vigorous re-establishment after coppicing	*S. viminalis, S. eriocephala* Michx.	Canada	Sennerby-Forsse and Zsuffa, 1995

2.2 General description of the zero-discharge willow system

ZDWS were developed in Denmark as a solution to wastewater treatment for sites where ground infiltration was not possible due to low subsoil permeability, a high water table or groundwater sensitivity (Brix and Arias, 2011). A system with no discharge was required to meet strict environmental standards. The earliest ZDWS were established in 1992 (Ministry of Environment and Energy, 2001a). At least 3,800 willow evapotranspiration (ET) systems are now in operation in Denmark, serving both single houses and communities of up to 73 houses (Brix and Arias, 2011).

Danish guidelines for the construction and installation of these systems were published in 2003 (Ministry of Environment and Energy, 2003). Briefly, a standard ZDWS consists of a septic tank, followed by a pumped distribution to the willow wetland. The wetland is a large trench excavated from the ground; 1.5 m deep, 8 m wide and with 45° slopes. The actual length of each ZDWS is calculated using the design guidelines. The quantity of wastewater expected to enter the wetland is first estimated. This quantity is compared against a 20 x 20 km grid-square map of Denmark which gives the area demand per 100 m^3 of wastewater, depending on the local annual precipitation and reference evapotranspiration at the site. Comparison with this map allows the length of the willow wetland to be calculated. A ZDWS in Denmark has an area demand of 124-291 m^2 100 m^{-3} wastewater (Ministry of Environment and Energy, 2003).

The base and sides of the wetland are sealed with an impermeable HDPE or LDPE liner and a protective geotextile membrane. The excavated soil is then backfilled. At

least three different willow clones are planted in the early spring at a density of approximately 2 m^{-2}. Each clone is planted in complete rows, with a total of six rows per wetland. During the winter following establishment half of the willows are coppiced, with the other being half coppiced twelve months later. In Ireland the window for coppicing willows is December to mid-March (Caslin *et al*., 2010). After the second year a coppicing cycle of two, three or four years is chosen. Wastewater is pumped into the sealed wetland from the septic tank and disperses via an underground network of perforated pipes located in a distribution layer of gravel beneath the surface of the soil.

2.2.1 Trees used in ZDWS

Usually three different willow clones are used in a ZDWS (Ministry of Environment and Energy, 2003). Polyclonal willow SRC has been shown to have higher yield levels and lower susceptibility to rust infections when compared to monoclonal stands (Dawson and McCracken, 1995; McCracken and Dawson, 1998). Details of the willow hybrids used in previous ZDWS studies are shown in Table 2.2. Poplar has been trialled for use in evapotranspiration systems in Italy (Guidi, 2008) and Sweden (Dimitriou and Aronsson, 2010) with some success. However, the use of poplar for SRC in Ireland has presented problems with harvesting and also a restricted area of Ireland in which yield is successful (Caslin *et al*., 2010). Therefore, it is likely that the use of zero-discharge wetlands in Ireland will likely focus on willow as the sole tree species.

Table 2.2 Willow varieties used in previous ZDWS studies

Willow variety	Pedigree	Reference
Jorr	*S. viminalis* x *S. viminalis*	Gregersen and Brix, 2001
Björn	*S. schwerinii* x *S. viminalis*	
Tora	*S. viminalis* x *S. schwerinii*	
Tordis	*S. schwerinii* x *S. viminalis*	Curneen and Gill, 2014
Sven	*S. schwerinii* x *S. viminalis*	
Inger	*S. triandra* x *S. viminalis*	
Torhild	(*S. schwerinii* x *S. viminalis*) x *S. viminalis*	
Olof	(*S. schwerinii* x *S. viminalis*) x S*. viminalis*	Curneen and Gill, 2015

2.3 Previous use of ZDWS in Ireland

Limited published work is available on the use of ZDWS in Ireland. O'Hogain *et al.* (2010) examined the performance of a hybrid reedbed and willow bed combination in Colecott, Co. Dublin, receiving municipal wastewater from 60 p.e. For most of the first year of operation (September 2003 – August 2004) there was no discharge from a 90 m^2 willow bed. In the second year outflow did occur due to the input of surface water volumes which were far in excess of the design volumes. In Lucan, Co. Dublin, a willow bed receiving wastewater from three successive reedbeds achieved zero-discharge performance over a two-year monitoring period (O'Hogain *et al.*, 2011). On a larger scale, wastewater from Donard village, Co. Wicklow (population in 2005 = 250 people), was pumped from a large septic tank to an eight hectare willow SRC plantation. A five-year surface water and groundwater monitoring programme was carried out on the Donard site which showed zero discharge of effluent from the system (www.clearpower.ie).

Despite these successes with hybrid reedbed willow systems, O'Hogain *et al.,* (2011) cautioned that direct application of the Danish guidelines to an Irish setting was not appropriate due to differences in climate. For example, average rainfall in Denmark is approximately 725 mm (Frich *et al*., 1997) while in Ireland average annual precipitation is 1,230 mm (Walsh, 2012). Over a four-year monitoring period of thirteen ZDWS, designed according to the Danish guidelines, in Wexford, Limerick and Monaghan, overflow occurred in all sites during the winter (Curneen and Gill, 2015).

2.4 ZDWS water budget

2.4.1 ZDWS water balance calculation

Difficulties in achieving true zero-discharge operation in Ireland can be understood by examining the ZDWS water budget.

The water balance of a typical CW can be described as follows (US EPA, 2000):

$$\Delta S = Q + P - ET - D \qquad \textbf{Equation 2.1}$$

where ΔS = change in storage, Q = wastewater inflow, P = precipitation, ET = evapotranspiration, and D = wastewater outflow. In the case of the Danish ZDWS, there is no outflow and equation 2.1 simplifies to:

$$\Delta S = Q + P - ET \qquad \textbf{Equation 2.2}$$

The working principle of the Danish ZDWS is that on an annual basis the evapotranspiration from the wetland is equal to the input from wastewater and precipitation, resulting in no discharge (Ministry of Environment and Energy, 2003).

Assuming that on an annual basis there is no overall change in storage the water budget simplifies to:

$$ET = Q + P \qquad \textbf{Equation 2.3}$$

Therefore, the difference between annual precipitation and evapotranspiration from the willow bed is the amount of wastewater that can be loaded on to the system on an annual basis (Gregersen and Brix, 2001).

Evapotranspiration is the combination of evaporation and transpiration (Eamus *et al.*, 2006). Evaporation is the vapourisation of water and its removal from the evaporating surfaces of water, soil and vegetation (Allen *et al.*, 1998). Transpiration is the vapourisation of water in the intercellular air spaces of plant leaves and its loss through the stomata (Allen *et al.*, 1998).

In both evaporation and transpiration, energy is required for the conversion of liquid water to water vapour. The energy for this conversion comes largely from direct solar radiation and to a lesser extent from the ambient air temperature (Eamus *et al.*, 2006). Evapotranspiration will be higher on a warm, sunny day than on a cool, cloudy day (Allen *et al.*, 1998). The driving force to remove water vapour is the difference in water vapour pressure between the evaporating surface e.g., soil or leaf surface, and the surrounding atmosphere (Randerson *et al.*, 2012). As the vapour content of the air increases, evapotranspiration gradually slows down and will stop altogether if the saturated air is not removed. Thus, evapotranspiration is also influenced by the wind speed and the relative humidity (Allen *et al.*, 1998).

Annual evapotranspiration totals of 980 – 1,310 mm during the first year and 1,470 – 2,090 mm during the second year have been reported from six ZDWS in Denmark (Gregersen and Brix, 2001). In the UK and Ireland willow evapotranspiration rates are lower, with figures of 400 – 1,100 mm reported for the growing season (Stephens *et al*., 2001; Curneen and Gill, 2014). Differences in evapotranspiration rates between Ireland and other countries are, at least in part, due to higher relative humidity in Ireland (Gill *et al*., 2014).

2.4.2 ZDWS crop factors

Measurement of actual evapotranspiration is often difficult, requiring expensive large-scale field lysimeters (Allen *et al*., 1998). In practice, reference evapotranspiration is usually measured through the use of the Penman-Monteith equation or an evaporation pan. Actual evapotranspiration is then estimated by multiplying the reference evapotranspiration by a species-specific crop factor (Allen *et al*., 1998):

$$ET_c = k_c ET_o \qquad \textbf{Equation 2.4}$$

where ET_c = actual evapotranspiration, ET_o = reference evapotranspiration and k_c = crop factor. Crop factors incorporate specific crop characteristics e.g. stage of crop development and the effect of the crop on soil evaporation, and vary only slightly with climate which allows crop factors to be transferred between locations (Allen *et al*., 1998; Paranychianakis *et al*., 2006). The crop factor is generally set to have a maximum of 0.6 - 1.4 for a vegetation area of at least 2,000 m^2 (Allen *et al.*, 1998; Baille, 1999). In the case of small wetlands, such as ZDWS, or long strips of tall vegetation, crop factor values can exceed this limit by 100% or more (Allen, 1995;

Allen *et al.*, 1998; Stephens *et al.*, 2001). Irrigated willow stands have exhibited crop factors of 0.7-1.0 at the beginning of the growing season, 1.2-1.6 during mid-season and approximately 2.0-2.5 during the late season (Persson and Lindroth, 1994; Sugiura, 2009).

The "clothesline" and "oasis" effects contribute to such high evapotranspiration rates and crop factors from ZDWS (Brix and Arias, 2005). The clothesline effect occurs when tall wetland vegetation is surrounded by shorter cover. Transport of sensible heat (heat that will give rise to a temperature change) into the willow trees and transport of water vapour away is enhanced by siting the wetland broadside to the prevailing wind (Brix, 2003). Wetlands with taller vegetation in general have higher values of k_c and ZDWS will contain many trees > 4 m in height (Kadlec and Wallace, 2009). The oasis effect occurs when the wetland is surrounded by dry ground. As the warm, dry air, in equilibrium with this ground, flows over the wetland the willow trees experience enhanced evapotranspiration due to the transport of sensible heat into the wetland (Brix and Arias, 2005).

In addition, the reference evapotranspiration is calculated using a theoretical low-lying grass cover i.e. a one-dimensional surface. A ZDWS can be regarded almost as a 1-4 m high cube, which exposes a much larger surface area to sunlight and throughput of wind (Ministry of Environment and Energy, 2001a). This departure from the reference crop can contribute to the very high growing season crop factors that are a feature of ZDWS.

2.4.3 Differences in ZDWS water balance between Denmark and Ireland

For a given volume of wastewater, the sizing of a Danish ZDWS is dependent on the local annual reference evapotranspiration and precipitation levels (Ministry of Environment and Energy, 2003). Precipitation thus plays a crucial role in determining the final size of the wetland. Over four years in a Danish ZDWS study, average annual evapotranspiration of 1,310 mm was recorded (Ministry of Environment and Energy, 2001b). Of that total, 520 mm (40%) was from wastewater and 790 mm (60%) was from precipitation. In a separate Danish ZDWS study, 50-80% of the water in a ZDWS was rainfall (Ministry of Environment and Energy, 2001a).

Large differences in precipitation exist between Ireland and Denmark. In Denmark, the average annual precipitation is 725 mm with values ranging from 550 mm in the southeast to 900 mm in the western interior (Frich *et al.*, 1997). Reference evapotranspiration ranges from 560 to 610 mm. The Danish ZDWS guidelines assume that evapotranspiration from the ZDWS is 2.5 times the annual reference evapotranspiration (Ministry of Environment and Energy, 2003), which gives a large precipitation deficit for the use of these systems in Denmark. Such a large precipitation deficit does not exist for the use of ZDWS in Ireland. The average annual rainfall in Ireland is 1,230 mm, ranging from 800 mm in the east to over 2,000 mm in the west (Walsh, 2012). Reference evapotranspiration rates in Ireland are lower than in Denmark, ranging from 390 mm to 560 mm, with the highest rates occurring in the south-east and the lowest in the north-west (Mills, 2000). Annually, only 28-56% of precipitation is lost as evapotranspiration (Mills, 2000).

Considering the higher precipitation rates in Ireland, it is likely that the area demand per unit of wastewater entering the wetland will increase for ZDWS in Ireland. The large footprint required for the use of ZDWS is a major constraint on the use of these systems in Ireland, both for cost and site size restrictions (Dubber *et al.*, 2014). Estimated capital costs for a ZDWS in Ireland are approximately €5,300 per capita (approx. €21,200 for a four person household), mainly due to the size of the systems and the related costs of the excavation work, liner purchase and installation (Dubber and Gill, 2014). In Denmark capital costs are approximately €8,000 in total depending on the site size (Brix and Arias, 2011).

The use of ZDWS in Ireland thus faces challenges in terms of installation costs and area demand. Improvements which lower the wastewater or rainwater input could lower the area demand for the wetland. This would make the system more suited to Irish conditions. The use of a polytunnel covering to prevent precipitation input is a potential way of lowering the area demand. An experiment to investigate this was considered appropriate.

2.4.4 Winter evapotranspiration

During the growing season, transpiration from willow stands in the open can be up to 80% of the total seasonal evapotranspiration (Persson and Lindroth, 1994; Persson, 1995; Białowiec *et al*., 2007b). In the northern hemisphere, peak transpiration rates for willows occur from July onwards and begin to reduce in September (Guidi, 2008; Pistocchi *et al.*, 2009). This decrease is due to reduced solar radiation to drive evapotranspiration, lower air temperatures, leaf senescence and the onset of plant dormancy (Allen *et al.*, 1998). Willow crop factors during

winter drop to approximately 1.0, with winter willow evapotranspiration totals of approximately 100 mm in Ireland (Curneen and Gill, 2014). This level of evapotranspiration is within the winter reference evapotranspiration rates of 76 – 130 mm for Ireland as calculated by Mills (2000). Due to the very low winter evapotranspiration rates from ZDWS, during this time period the function of the ZDWS is to accumulate and store all incoming wastewater and precipitation (Ministry of Environment and Energy, 2003). This low rate of winter evapotranspiration, in combination with a constant level of wastewater input throughout the year and high winter precipitation, leads to the large size of these systems.

In conventional wetland "filter" treatment systems designed for cold climates, the focus is on reducing energy loss to the atmosphere. This is achieved through the use of mulching and the avoidance of open water which loses energy through evapotranspiration, as this represents energy loss that could otherwise be used for treatment purposes (Wallace *et al.*, 2001). In contrast, in a ZDWS the focus is on maximising evapotranspiration even during the winter months.

To increase the evapotranspiration rates during the winter months, the use of winter-active species could be considered. Winter heliotrope, *Petasites fragrans* (Vill.) C. Presi, is a winter-active member of the Asteraceae exhibiting year-round growth in Ireland (O'Mahony, 2009). Originating in North Africa, *P. fragrans* was introduced to Ireland in the nineteenth century. *P. fragrans* flowers from November to January (O'Mahony, 2009). Only male plants of this dioecious species were introduced and in Ireland *P. fragrans* spreads rhizomatously (Parnell and Curtis,

2012). Due to its method of dispersal *P. fragrans* forms dense carpets about 30 cm in height (National Roads Authority, 2010). Figure 2.1 shows the distribution of *P. fragrans* across Ireland, indicating coverage over much of the island. *P. fragrans* is shade-tolerant and can colonise both damp and dry soils (Devlin, 2014). As part of the current research, this year-round growth indicated its potential for use as a low-lying winter-active ground flora under willow as part of a ZDWS. However, due to its status as an invasive species (Stokes *et al.*, 2006), precautions should be taken when working with *P. fragrans* to avoid further dispersal.

The combination of willow species with other plant/tree species has previously been studied in intercropping systems (Table 2.3). Attention has been focussed primarily on increasing the biomass production of the willow species in the intercropped system. However, there is no published literature on the combination of willows with winter-active species to increase winter evapotranspiration. The use of winter-active species as a novel way of increasing system efficiency was, therefore, considered worthy of investigation.

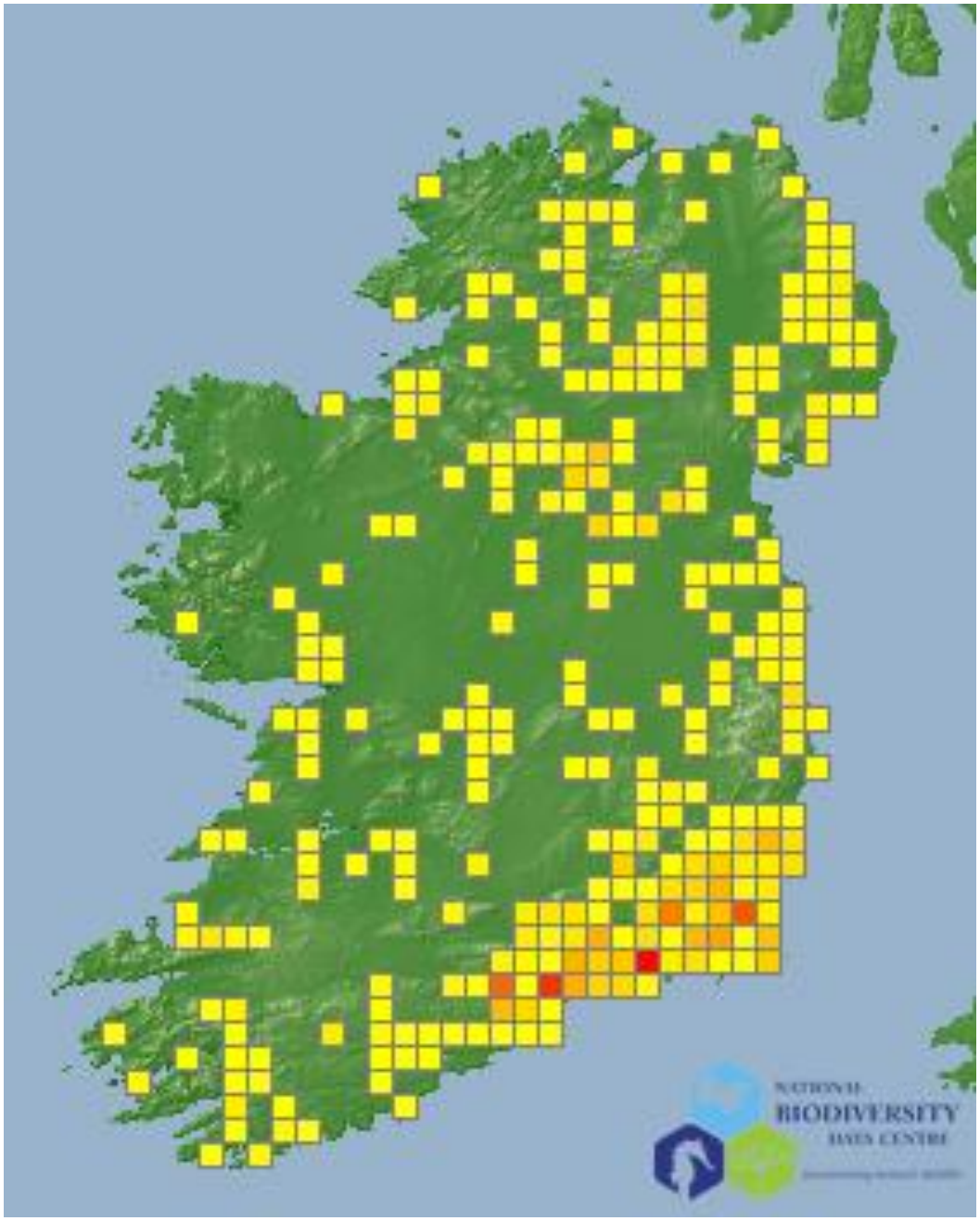


Figure 2.1 *P. fragrans* distribution across Ireland. Image: National Biodiversity Data Centre, 2014. Coloured boxes indicate areas of *P. fragrans* occurrence: yellow = 1 – 6 records per 10 km square; orange = 7 – 22 records per 10 km square, red = > 23 records per 10 km square.

Table 2.3 Willow cropping with other tree/plant species.

Willow species	Intercrop species	Function	Country	Reference
S. dasyclados, S. miyabeana, S. purpurea	*Julglans nigra* L., *Quercus rubra* L., *Fraxinus americana* L., *Robinia pseudoacacia* L.	Improvement of willow growth through combination of willows with intercrop species in agroforestry system	Canada	Clinch *et al*., 2009
Salix sp.	*Prunus avium, Populus* sp.	Biomass, timber production, ecosystem services	Ireland, Northern Ireland	Lunny *et al*., 2014
Salix sachalinensis F. Schmidt, *Salix discolor* Muhl.	*Trifolium repens* L.	Green manure to increase willow yield	USA	Arevalo *et al*., 2005
Salix miyabeana Seemen.	*Caragana arborescens* Lam.	Increase willow yield on poorly productive sites	Canada	Moukoumi *et al*., 2012
Salix caprea L.	*Arabidopsis halleri subsp. halleri* L.	Phytoextraction of Zn and Cd	Austria	Wieshammer *et al*., 2007

2.4.5 The use of a wetland cover to reduce hydraulic load

To reduce the overall hydraulic loading into a ZDWS, Curneen and Gill (2015), modelled the use of a theoretical impermeable membrane, placed on the surface of the wetland soil, finding that, if 60% or more of the annual rainfall could be diverted, the system could be fully zero-discharge. The use of a polytunnel cover on an evapotranspiration bed in New Zealand has been proposed by Balley and Dakers (1996) due to the precipitation during the winter months exceeding the Penman evapotranspiration. The authors suggested the use of a removable transparent plastic roof for placement over the evapotranspiration system during winter to

prevent rainwater entering. The installation of a permanent polytunnel covering to prevent any rainfall entering simplifies equation 2.3 to:

$$ET = Q \qquad \textbf{Equation 2.5}$$

This increases the wastewater evapotranspiration efficiency, such that 100% of the evapotranspiration comprises wastewater. Theoretically, the unit area demand for a ZDWS should decrease, as precipitation, which had comprised 50-80% of the total evapotranspiration, is removed from the area calculation.

However, overall evapotranspiration can be lowered due to the presence of a polytunnel. Irrigated crops within polytunnels and glasshouses have lower evapotranspiration than outdoor crops due to the low evaporative demand indoors (Orgaz *et al*., 2005; Grant *et al*., 2012). Despite higher temperatures, reference evapotranspiration is reduced in polytunnels to as low as 56% of the outdoor value due to lower wind speed, higher relative humidity and lower incident solar radiation (Fernandes *et al.*, 2003). Solar radiation is considered the meteorological variable determining most of the ET_o and polytunnels can reduce solar radiation by 39% compared to outdoors (Fernández *et al.*, 2010).

The principal cause of glasshouse heating is the suppression of natural convection within the structure which traps sensible and latent heat (Bello, 1982). Air movement is much reduced inside polytunnels with wind speeds inside measured at 0.1 – 0.3 m s^{-1}, compared to 1.5 – 3.0 m s^{-1} outdoors (Fernández *et al.*, 2010). Reduced wind speed leads to an increase in boundary layer thickness around the plant canopy resulting in lower diffusion rates and an overall reduction in evapotranspiration (Eamus *et al.*, 2006). As a result, the boundary layer

conductance for glasshouse-grown crops is much lower than the same crops grown outdoors (Boulard and Wang, 2000). Relative humidity is higher inside polytunnels (65 – 80% inside, 60-70% outside)(Fernández *et al.*, 2010) which reduces evapotranspiration due to a smaller vapour pressure difference between the evaporating surface (leaf) and the atmosphere (Eamus *et al.*, 2006).

With regard to increasing efficiency of wastewater removal on a per unit area basis, a trade-off thus exists between eliminating precipitation loading to the ZDWS through the use of a polytunnel cover and reducing the evapotranspiration rate due to lower evaporative demand. With the objective of increasing ZDWS efficiency, this trade-off warranted investigation.

2.5 The effects of coppicing regime and soil type on ZDWS performance

In addition to attempts to maximise evapotranspiration from ZDWS, both the coppice management regime and the soil type used are important factors in system performance. Both of these factors can influence wastewater loss and nutrient removal from the system. These are reviewed below in the light of ZDWS wastewater removal and treatment performance.

2.5.1 Coppicing regime

Coppicing is the regular cutting back of trees to near ground level to remove apical dominance and encourage faster growth and increased yields (Rooney *et al.*, 2009). Willow is an ideal plant species for short rotation coppice (SRC) due to its vigorous ability to re-sprout (Dawson, 2011). Willows in a ZDWS are usually coppiced in a three- to four-year cycle as this cycle has been shown to maximise yield in SRC (Adegbidi *et al*., 2001; Szczukowski *et al*., 2005; Wang and MacFarlane, 2012).

Willows can produce yields greater than 14 odt ha^{-1} yr^{-1} (oven dry tonnes per hectare per year) even after eighteen years, comprising five three- to four-year coppice cycles (Guidi *et al.*, 2013).

In the case of using a wetland with a polytunnel cover, annual harvesting is required to prevent damage to the polytunnel which is usually 2-2.5 m in height, as willow regrowth height can be 2-3 m in the first year after coppice (Caslin *et al*., 2010). In general, annual harvests have lower yields than longer harvest cycles (Szczukowski *et al*., 2002, 2005; Stolarski *et al*., 2008). Triennial harvests in the USA had significantly higher biomass production than the sum of three individual annual harvests (65 and 23 odt ha^{-1}, respectively (Kopp *et al*., 1997). In the same study, yield at the end of the second biennial cycle was significantly greater than the sum of the third and fourth years of the annual harvests. For three poplar cultivars the same trend was evident with the yield for a single four-year harvest in the UK being higher than the yield from two two-year harvests (Armstrong *et al.*, 1999).

In addition to effects on yield, coppicing can also impact upon survival. Kopp *et al.* (2001) recorded continuous decline during ten years of annual coppicing to 46% survival. In contrast, after five growing seasons for willows under annual, biennial and triennial coppice cycles, survival of willow trees was not significantly different between the three coppicing cycles (Kopp *et al.*, 1997). In a wetland polytunnel system where annual coppicing must be carried out, the impacts of this cycle length on willow survival and growth, and hence evapotranspiration, need to be investigated.

Willow stem nutrient concentration increases with shorter harvest cycle length (Hytönen, 1995). This is due to the higher bark : wood ratios, with the bark of younger stems having higher nutrient concentration than that of older stems (Adegbidi *et al.*, 2001). *S. alba* grown under a one-year rotation in the USA removed 46 kg N ha^{-1} yr^{-1} and 6 kg P ha^{-1} yr^{-1}, but these figures fell to 30 kg N^{-1} ha^{-1} yr^{-1} and 3.8 kg P ha^{-1} yr^{-1}, respectively, under a three-year cycle (Adegbidi *et al.*, 2001). In the same study the nutrient use efficiency (kg biomass produced per kg nutrient absorbed) increased from year one to year three for both nitrogen and phosphorus. In the context of nutrient management, annual harvesting of willows will lead to the highest amount of nutrients removed from the ZDWS (Adegbidi *et al*., 2001).

2.5.2 Soil type

Maximising yield is important in the context of ZDWS, as transpiration is directly related to biomass growth (Licht and Isebrands, 2005). Typical yields from willow SRC plantations in Ireland and the UK are 7 - 15 odt ha^{-1} yr^{-1} (McElroy and Dawson, 1986; Cannell *et al*., 1987; Rice, 2001; Larsson *et al*., 2003; Wickham *et al*., 2010; Dawson, 2011).

ZDWS are typically installed in areas where subsoil percolation rates are low (Brix and Arias, 2011). This is generally due to a high clay content in the soil. Soils of up to 20% clay and 20% silt have been used in ZDWS in Denmark (Ministry of Environment and Energy, 2001a). In county Wexford, zero-discharge willow systems have been trialled in areas of heavy clay “marl” subsoil (Curneen and Gill, 2012). Due to the permeability issues caused by subsoils high in clay content, it is likely

that subsoil clay content will be a leading determinant of where ZDWS are likely to be sited.

Many studies have found higher production in clay soils, both in field trials and pot/lysimeter studies (Table 2.4, studies 1-4). In contrast, other studies have found higher growth in sandy soils (Table 2.4, studies 5-6). With differences in growth reported between different soil types, investigating the growth of willows across a range of soil types in Ireland is warranted.

In the UK, across five soil types (sand, sandy loam, sandy clay loam, silty loam and clay), annual willow evapotranspiration did not differ significantly and varied between 430 and 480 mm (Stephens *et al.*, 2001). In a lysimeter study involving two subsoil types (49% clay and 1% clay), during the growing season in Sweden, *S. schwerinii x S. viminalis* willows in the high clay subsoil had average daily evapotranspiration rates of 6.1 mm day^{-1}, compared to 4.8 mm day^{-1} for willows in the low clay subsoil (Dimitriou and Aronsson, 2011). Water use efficiency (WUE) can also be affected by soil type, with reported willow WUE of 2.57 g kg^{-1} on sand in Poland (Białowiec *et al*., 2007a), to 5.5 g kg^{-1} on clay in Sweden (Lindroth *et al*., 1994). Knowledge of the soil type and its likely impact on willow growth and transpiration is important to allow for ZDWS to be sized appropriately for the soil type in each location.

Table 2.4 Willow yield in relation to soil/subsoil type

Study number	Willow species/variety	Soil/subsoil type	Yield (DW = dry weight)	Country	Reference
1	*S. viminalis* 78183	Clay (47% clay) Sand (4.5% clay)	15 kg DW m^{-2} 4.5 kg DW m^{-2}	Sweden	Dimitriou and Aronsson, 2004
2	Tora (*S. schwerinii* x *S. viminalis*)	Clay (47% clay) Sand (4.5% clay)	2.5 kg DW m^{-2} 0.8 kg DW m^{-2}	Sweden	Dimitriou and Aronsson, 2011
3	*S. viminalis* 5027	Clay (49% clay) Sand (13% clay)	70.36 odt ha^{-1} 40.72 odt ha^{-1}	Canada	Labrecque and Teodorescu, 2003
	S. discolor Mühl.	Clay (49% clay) Sand (13% clay)	31.48 odt ha^{-1} 25.43 odt ha^{-1}	Canada	Labrecque and Teodorescu, 2003
4	*S. viminalis* 78183	Clay Sand	21-22 odt ha^{-1} yr^{-1} 9-10 odt ha^{-1} yr^{-1}	Sweden	Rytter, 2001
5	*S. nigra* Marshall.	Fine-textured sediment Coarse-textured sediment	0.09 kg DW $plant^{-1}$ 1.25 kg DW $plant^{-1}$	USA	Schaff *et al*., 2003
6	Jorr (*S. viminalis*)	Heavy clay Sandy loam	5.73 kg DW $plant^{-1}$ 10.74 kg DW $plant^{-1}$	UK	Martin and Stephens, 2006

When comparing soil types, differences in nutrient removal are apparent. These differences can be due to changes in expression as nutrient concentration (%) or total nutrient content (kg ha^{-1} removed). In Canada, Labrecque and Teodorescu (2003) recorded higher nitrogen and phosphorus concentrations in *S. viminalis* trees grown in clay soil, compared to sandy soil. However, in general, nutrient concentration does not vary with substrate, with differences in removal rate due to differences in nutrient content. In Sweden, annual uptake of nitrogen was 193 kg N ha^{-1} yr^{-1} in willows grown in clay compared to 97 kg N ha^{-1} yr^{-1} in willows grown in

washed quartz sand, with the differences due to increased yield on the clay soil rather than substrate-induced changes in nitrogen allocation (Rytter, 2001). Soil type can also affect phosphorus removal in the harvestable willow biomass. Removal rates have ranged from 6 – 9 kg P ha^{-1} yr^{-1} in clay soil to 10-11 kg P ha^{-1} yr^{-1} in sandy soil (Adegbidi *et al.*, 2001; Larsson *et al.*, 2003).

2.6 The use of mycorrhizal fungi to improve willow performance

Many factors affect willow establishment success including duration of storage of willow cuttings, time of planting, competition from weed species and the level of hydraulic loading during the first year of operation (Ministry of Environment and Energy, 2003; Volk *et al*., 2004; Curneen and Gill, 2012). Good establishment of willow cuttings is critical to the long-term success of willow SRC and ZDWS (DEFRA, 2004; Curneen and Gill, 2012). To aid in plant establishment, the use of mycorrhizal fungal inoculum has become widespread in the land reclamation and horticultural industries (Davies, 2000; Quoreshi and Khasa, 2008; Mummey *et al*., 2009). Inoculation is carried out so that plants benefit from the introduced mycorrhizal fungi in addition to the indigenous mycorrhizal fungi in the soil (Wijesinghe, 2012).

Mycorrhizal fungi engage in a mutualistic symbiosis with plant roots (Brundrett *et al.*, 1996). Over 80% of plant species form mycorrhizal associations (Smith and Read, 2008). The symbiosis is characterised by the bi-directional exchange of resources at the plant-mycorrhiza interface (Bucking *et al.*, 2012). The mycorrhiza aids in nutrient uptake and supply to the plant as the hyphae extend beyond the nutrient depletion zone that develops around plant roots (Rooney *et al.*, 2009). In

return, the plant supplies photosynthetically fixed carbon to the mycorrhizal symbiont (Bonfante and Genre, 2010).

2.6.1 Types of mycorrhizal fungi

At least seven different types of mycorrhizal association have been described, including ericoid, arbutoid, monotropoid, ectendo- and orchid mycorrhiza (Brundrett *et al.*, 1996). Only the two most common will be considered in this thesis: arbuscular mycorrhizal fungi (AMF) and ectomycorrhizal fungi (ECM). AMF belong to the Glomeromycota phylum and are the most common mycorrhiza type (Smith and Read, 2008). AMF colonise intracellularly within the root cortex with the arbuscules acting as the main point of nutrient exchange between the host plant (Brundrett *et al.*, 1996). Extraradical hyphae extend outwards from the plant root surface into the rhizosphere. Vesicles located within the plant root serve as storage structures (Davies, 2000). ECM are predominantly formed on woody perennials (Smith and Read, 2008). ECM belong to the Basidiomycota and Ascomycota phyla (Brundrett *et al.*, 1996). The hyphae of ECM colonise intercellularly in the root cortical cells and form a network called the Hartig net which is the main point of nutrient exchange (Davies, 2000). On the outside of the root, the fungus forms a sheath or mantle that encases the root with the external mycelium penetrating outwards into the soil (Smith and Read, 2008). Ectomycorrhizal fungi are most predominant on the root tips, but can also occur along the unsuberised parts away from the root tips (Püttsepp *et al.*, 2004).

Willow is one of the few tree genera that is dual-mycorrhizal and can associate with both AMF and ECM (Trowbridge and Jumpponen, 2004; Parádi and Baar, 2006).

Other dual mycorrhizal genera include poplar, eucalyptus and alder (Becerra *et al*., 2009; Karliński *et al*., 2010; Kariman *et al*., 2012). In natural settings, rates of ECM colonisation for willow are generally higher than AMF colonisation (Hashimoto and Higuchi, 2003). The difference can be large, with less than 1% root length colonisation by AMF and 19.4% colonisation by ECM (Trowbridge and Jumpponen, 2004). In contrast, Sasaki *et al*. (2001) recorded AMF colonisation levels of 30-90% on the roots of *S. gracilistyla* Miq.*, S. chaenomeloides* Kimura and *S. subfragilis* Anders in Japan. *S. viminalis* is known to show low levels of ECM colonisation in comparison to other woody species (Baum *et al.*, 2002a). Other dual-mycorrhizal trees, such as poplar, have shown higher rates of AMF colonisation than ECM colonisation (Khasa *et al.*, 2002).

Soil disturbance can adversely affect mycorrhizal fungi (Evans and Miller, 1988). As the soil is considerably disturbed during construction, levels of indigenous mycorrhiza resident in the soil may be low after ZDWS installation. The lack of mycorrhizal fungi within the soil is a leading cause of poor plant establishment and growth in forestry sites (Ortega *et al.*, 2004). Arable soils usually contain AMF since the majority of arable crops can associate with AMF, but ECM levels can be lower due to the long-term absence of suitable hosts (Baum *et al*., 2009). Where ZDWS are installed on formerly arable soils, the low level of ECM resident in the soil could impact on the willow performance. Brundrett (1991) noted that in some cases it may be beneficial to introduce mycorrhizal fungi to replace lost inoculum.

The effects of AMF and ECM on establishment rates, plant nutrition, biomass growth and disease resistance in willow and other species are reviewed below.

2.6.2 The effects of mycorrhizal fungi on establishment and biomass production

Inoculation of *S. repens* with the ECM *Hebeloma leucosarx* P.D. Orton and the AMF *Glomus mosseae* Gerd & Trappe led to a 90% survival rate (irrespective of mycorrhizal species) compared to 50% survival in non-inoculated controls (van der Heijden, 2001). Survival rates (after two growing seasons) of 92% for *S. viminalis* trees inoculated with the AMF *Glomus intraradices* Schenk & Smith compared to 86% for the control were recorded by Bisonnette *et al.* (2010). Both *Quercus virginiana* Mill. and *Sapium sebiferum* L. have shown significantly increased survival after transplanting when inoculated with AMF and ECM (Davies and Call, 1990).

For *Salix repens*, inoculation with the AMF *G. mosseae* was more beneficial in the short time after establishment (12 weeks) for shoot and root growth, but in the longer term (30 weeks) the ECM *H. leucosarx* was more beneficial (van der Heijden, 2001). Fillion *et al.* (2011) found that inoculation with the AMF *G. intraradices* led to an increase in initial plant growth during the establishment phase. Inoculation of the ECM *Thelephora terrestris* Ehrh. onto *S. viminalis* led to significantly higher growth compared to non-inoculated trees over the first 98 days due to a rapid uptake of phosphorus during the first 50 days (Jones *et al.*, 1991). Of ten ECM species inoculated onto *S. repens*, *Cotinarius trivialis* J. E. Lange*, H. leucosarx* and *H. psammophilum* Bon caused the biggest increase in shoot dry weight (van der Heijden and Kuyper, 2003). In contrast, Lefebvre (2007) found no difference in biomass production between *S. viminalis* trees inoculated with the AMF *G. intraradices* and non-inoculated controls. Poplar can benefit from mycorrhizal inoculation during the year of establishment, with inoculated plants performing

better than non-inoculated controls (Baum *et al.*, 2002b; Quoreshi and Khasa, 2008). *Populus trichocarpa* cv. Weser cuttings, inoculated with the ECM *Laccaria bicolor* (Maire) P.D. Orton or the ECM *Paxillus involutus* (Batsch) Fr., showed greater shoot dry weight production after six months of growth in a nitrogen- and organic matter-rich soil in comparison to non-inoculated controls (Baum *et al.*, 2000).

2.6.3 The role of mycorrhizal fungi in disease resistance and stress tolerance

Mycorrhizal root colonisation can improve resistance to disease for both belowground and aboveground parts of the plant. The fungal sheath of ECM forms a physical barrier to root pathogens (Davies, 2000). AMF and fungal root pathogens compete for infection and colonisation sites and the plant chemical defence compounds induced by AMF symbiosis confer localised and systemic resistance (Smith *et al.*, 2010). Rust (*Melampsora* spp.) is a major pathogen of willow and poplar trees (McCracken and Dawson, 1998). In mycorrhized poplar trees, the presence of ECM can lead to reduced negative effects of rust (Pfabel *et al.*, 2012). In mycorrhized *Pinus tabulaeformis* Carr. seedlings, colonisation by the ECM *Amanita vaginata* (Bull.: Fr.) Vitt. led to increased protection from damping off (Zhang *et al.*, 2011).

The presence of mycorrhizal fungi can lead to greater stress tolerance in the host plants. In *Salix x dasyclados* inoculated with the ECM *Paxillus involutus,* increased zinc uptake occurred without a detrimental effect on plant growth (Baum *et al*., 2006). Inoculation with mycorrhizal fungi has been used to increase plant growth in the presence of heavy metal contamination (Fayuan Wang *et al*., 2005; Lingua *et al*., 2008). Zinc-adapted *Suillis bovinus* (L.) Roussel (from a zinc-contaminated site)

conferred greater protection from zinc stress upon *Pinus sylvestris* L. compared to normal wild type *S. bovinus* (Adriaensen *et al*., 2004). AMF can lead to increased salt stress tolerance of the host plant through the adjustment of salt-induced osmotic imbalance by the accumulation of osmolytes (Kapoor *et al*., 2013).

In the absence of wastewater application during the first year, the soil may suffer from low water availability during a particularly dry summer. Mycorrhizal associations can aid drought tolerance of the host plant. Mycorrhizal fungi chemically enmesh and stabilize soil microaggregates into macroaggregates through the production of the glycoprotein glomalin (Augé, 2004). This aggregation leads to increased water availability in the pores of the soil, which can be very useful in the establishment of willow plantations where irrigation is not possible. Where soil water availability is low, mycorrhizal fungi can increase the production of extraradical hyphae which allows for increased water uptake (Davies, 2000).

2.6.4 The effects of mycorrhizal fungi on nutrient and water uptake during establishment

Nutrient application is generally not recommended on most willow SRC during the first growing season as weed control is imperative and fertiliser application may exacerbate the weed problem (Caslin *et al.*, 2010). In ZDWS, where willows have been planted in autumn, standing water during the first growing season has limited the establishment of the willow trees (Ministry of Environment and Energy, 2001a). Delaying wastewater input to the ZDWS until after the first growing season to avoid hydraulic overloading of the willow seedlings is advised (Curneen and Gill, 2012). In the absence of nutrient input from wastewater application during the first year, soil nutrient levels may be low.

Mycorrhizal hyphae can extend beyond the nutrient depletion zone around roots allowing for increased nutrient uptake. ECM have stronger influence on nitrogen nutrition, while AMF have a stronger influence on phosphorus nutrition (Smith *et al.*, 1994). Mycorrhizal fungi can improve the growth of willows by as much as two-fold compared to non-mycorrhized willows (Jones *et al.*, 1991). An increase in biomass production is often due to an increase in phosphorus use efficiency (Fillion *et al.*, 2011). The ECM *Hebeloma syrjense* P. Karst. can improve nutrition of willows when organic sources of nutrients are added to the soil (Tibbett and Sanders, 2002). *S. viminalis* inoculated with the ECM *L. proxima* (Boud.) Pat. or the ECM *T. terrestris* had a positive effect on shoot growth, and a higher rate of phosphorus uptake, when grown in a low phosphorus soil, compared to non-mycorrhizal willows (Jones *et al.*, 1990). Up to 33% higher stem P content has been found in *S. viminalis* inoculated with the AMF *G. intraradices* compared to control willows (Fillion *et al.*, 2011). Phosphorus uptake rates were more than eight times greater in mycorrhized pine seedlings compared to control seedlings (Colpaert *et al.*, 1999).

It is generally accepted that fertilisation reduces the mycorrhizal root colonisation, although sparse evidence exists to support this in willow SRC (Rooney *et al.*, 2009). Above 10 mg extractable P kg^{-1}, Jones *et al.* (1990) recorded a reduction in mycorrhizal colonisation of *S. viminalis* by the ECM *L. proxima*, and a reduction above 21 mg extractable P kg^{-1} for the ECM *T. terrestris.* However, Fillion *et al.* (2011) reported that increasing P irrigation from 0.5 mg L^{-1} to 10 mg L^{-1} led to an increase in root colonisation. Nitrogen fertilisation on nitrogen-poor soils also increased ECM colonisation and AMF spore density (Baum *et al.*, 2002a).

In general, colonisation by AMF usually results in an increase in transpiration or stomatal conductance (Allen *et al.*, 1981; Augé, 2001, 2004; Augé *et al.*, 2004). This indicates that the use of AMF for ZDWS, where the objective is to increase evapotranspiration rates, may be beneficial. ECM can cause an increase in plant water uptake. *Pinus radiata* D. Don trees inoculated with the ECM *Rhizopogon roseolus* (Corda ex Sturm) Th. Fries showed higher hydraulic conductance at the soil-root interface compared to non-inoculated controls (Ortega *et al.*, 2004).

2.6.5 The effects of wastewater application on mycorrhizal fungi

The application of wastewater to soil is known to alter the microbial community (Filip *et al.*, 2000). After 43 years of irrigation, soil irrigated with freshwater showed a higher AMF diversity (Shannon diversity index, H = 2.40) than soil irrigated with secondary treated municipal wastewater (H = 1.94) (del Mar Alguacil *et al.*, 2012). Soils receiving municipal wastewater irrigation for ninety years had lower AMF spore abundance than soils receiving the same irrigation for only five years (Ortega-Larrocea, 2001). Short periods of wastewater irrigation (< two years) do not significantly alter mycorrhizal fungi presence in willow SRC (Truu *et al.*, 2009). However, with increasing length of irrigation, species of *Glomus* began to dominate the mycorrhizal fungi population leading to decreased mycorrhizal fungal diversity (Ortega-Larrocea *et al.*, 2007).

2.7 Research Motivations and Objectives

The motivation for this research was borne out of the pressing need to protect groundwater and surface water quality in Ireland under the Water Framework Directive. The creation of an OWTS for sites of very low water permeability is of

great importance, given the prevalence of these soil conditions across Ireland. Despite this importance, O'Hogain *et al.* (2010) advised that the Danish guidelines for ZDWS cannot be directly applied to Ireland due to differences both in climate and in wastewater production. To date, the work of Curneen and Gill (2015) is the only research published on the use of field-scale ZDWS in Ireland. The dearth of evidence on the effectiveness of ZDWS in Ireland makes it challenging for planning authorities to approve the use of these systems as part of planning applications, as the systems are not included in the Code of Practice (EPA, 2009).

Based on the literature reviews outlined in chapters 1 and 2, there are two general aims of this research:

a) to increase the body of evidence for the use of ZDWS in Ireland;
b) to explore a number of methods to increase system efficiency and reduce area demand.

In order to realise the above aims, the specific objectives of this research are:

i. to investigate the use of a polytunnel covered ZDWS to increase wastewater removal from a ZDWS by eliminating input from rainfall;
ii. to examine the potential use of a winter-active species, *P. fragrans*, to increase evapotranspiration in a ZDWS;
iii. to investigate the impact of annual coppicing and a gradient in soil clay percentage on willow evapotranspiration, growth and nutrient balance;

iv. to investigate the effect of a commercial mycorrhizal fungal inoculum on the growth of *S. viminalis* trees;

v. to explore the development of a willow-specific mycorrhizal fungal inoculum for use in ZDWS.

2.8 References

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Chapter 3 Monitoring of the water balance and willow growth in pilot-scale zero-discharge willow systems

3.1 Abstract

ZDWS were monitored over a two-year period in east County Cork, Ireland. Polytunnels were placed over the willow constructed wetlands as a novel design modification. Maximum temperature was significantly higher in covered unplanted wetlands compared to maximum temperatures outdoors. Wastewater removal by covered willow wetlands was high, but not significantly different from that by covered, unplanted wetlands. Evapotranspiration rates during the summer months were lower than rates reported elsewhere in Ireland. Highest crop factor values were found during the autumn indicating that the main benefit of the cover on the crop factor occurs during this time period when reference evapotranspiration is reducing following the summer maxima. While annual evapotranspiration rates were low, the actual amount of wastewater removed was high; wastewater comprised 100% of the liquid lost through evapotranspiration as the polytunnels intercepted incident rainfall. Despite the use of tertiary effluent as the wastewater, the amount of nutrients applied to the willow wetlands was sufficient for adequate growth, though biomass production and tree survival in the willow wetlands declined from year to year.

3.2 Introduction

The most common OWTS is the septic tank and percolation area system, accounting for 89.7% of these systems (Central Statistics Office, 2012). These systems are heavily reliant on the subsoil for pollutant attenuation (McKinley and Siegrist, 2011). The subsoil permeability plays a crucial role as this determines the ability of the soil to transfer wastewater away from the percolation area. Where the subsoil permeability is very low, effluent ponding on the soil surface above the percolation area can occur (McCarthy *et al*., 2009), leading to surface water pollution and public health concerns (Smyth *et al*., 2012).

The majority of subsoils in Ireland are unsorted glacial till which is of low to moderate permeability (Swartz *et al*., 2003). It is estimated that up to 39% of the land surface of Ireland will experience inadequate percolation for all or part of the year (EPA, 2012). In 2009, the Code of Practice published by the Environmental Protection Agency in Ireland introduced a maximum T-test value of 90 (k_{fs} = 0.047 m day^{-1}). The T-test is a falling head percolation test used to assess subsoil permeability. Above this maximum value discharge to ground is not acceptable as surface ponding will occur. Thus, a solution is required for sites where low subsoil percolation conditions exist.

To address this challenge, interest has turned to the use of the Danish ZDWS as a potential solution in areas of very low subsoil permeability (Dubber and Gill, 2014; Curneen and Gill, 2015). The working principle of the Danish ZDWS is that, on an annual basis, the evapotranspiration from the wetland is equal to the input from wastewater and rainfall combined (Ministry of Environment and Energy, 2003).

Annual evapotranspiration rates in excess of 1,000 mm have been reported for four varieties of *Salix viminalis* L. receiving primary effluent during the first growing season of a lysimeter study in Ireland (Curneen and Gill, 2014). Under field conditions, peak annual evapotranspiration rates of 930 mm have been reported from Co. Wexford (Curneen and Gill, 2015).

In a maritime climate such as Ireland, the possibility of making ZDWS truly zero-discharge has been questioned, as annual rainfall rates far exceed annual reference evapotranspiration (Curneen and Gill, 2015). In Ireland, rainfall ranges from 800 mm in the east to 2,000 mm in the west (Walsh, 2012). In comparison, rainfall in Denmark ranges from 550 mm in the southeast to 900 mm in the west (Frich *et al*., 1997). Reducing the hydraulic loading from rainfall into a ZDWS by 60% has been postulated as the level of reduction required to make the system fully zero-discharge on an annual basis in Ireland (Curneen and Gill, 2015). An investigation into the use of a polytunnel cover to prevent rainfall entering the ZDWS is therefore warranted.

As willows can grow 2-3 m in height in the first year (Caslin *et al*., 2010), the use of a polytunnel over a ZDWS for this purpose will necessitate annual coppicing of the willow crop to prevent damage to the polytunnel structure. Annual harvests of willow SRC have been shown to lead to lower yields compared to longer harvest cycles, with 3 to 4 year harvest cycles found to be optimal (Szczukowski *et al*., 2002, 2005; Stolarski *et al*., 2008). In addition to effects on yield, annual coppicing can also impact negatively upon willow tree survival. After ten years of annual coppicing, survival can be as low as 46% (Kopp *et al*., 2001). In contrast, after five

growing seasons for willows under annual, biennial and triennial coppice cycles, survival of willow trees was not significantly different between the three coppicing cycles (Kopp *et al*., 1997). The impact of annual coppicing on both yield and survival thus warrants further examination.

3.2.1 Objectives

The objectives of the research described in this chapter are:

(a) to determine the water balance of pilot-scale polytunnel-covered ZDWS receiving tertiary effluent.

(b) to determine the growth of *S. viminalis* undergoing annual coppicing as part of a polytunnel-covered ZDWS.

3.3 Materials and methods

3.3.1 Site description and construction

The field site was located on the grounds of Cloyne wastewater treatment plant (WWTP) in east County Cork (W910677) (Figure 3.1). The facility was constructed in 1995 and provides tertiary wastewater treatment to Cloyne village (p.e. = 1,400). Cloyne WWTP is operated by the EPS Group for Cork County Council under a ten-year operation contract (EPA and Cork County Council, 2010). This site was chosen due to the continuous availability of municipal wastewater. Furthermore, in a "contained" polytunnel system, tertiary effluent is the effluent most likely to gain approval for use. Wastewater is treated in four stages: an aeration tank, a clarifier, a picket fence thickener (PFT), and a reed bed. Screened wastewater is biologically treated by activated sludge in the aeration tank. Wastewater and sludge flow from

the aeration tank to the clarifier by gravity. Returned sludge from the clarifier also enters the aeration tank. Sludge flows from the clarifier to the PFT, from which it is removed twice monthly. Supernatant from the clarifier flows to the reed bed through a splitting chamber. The wastewater receives tertiary treatment in the reed bed. Treated effluent is discharged to the adjacent Spidal stream as per the discharge licence D0298-01 issued by Cork County Council in 2010.

3.3.1.1 Construction of zero-discharge wetlands

Construction work began on the field site during June 2007 before the start of the research project described in this thesis. Twelve zero-discharge wetlands were excavated by a mechanical digger in a plot of land adjacent to the reedbed (Figure 3.1). Wetlands were approximately 11 m long x 2.2 m wide x 1.2 m deep (exact surface area dimensions are shown in Table 3.1). Soon after starting the experimental period it was noticed that the water levels in wetlands 1, 3, 6 and 11 approximately followed that of the surrounding water table. It was assumed that these wetlands were leaking and, as a result, these wetlands were not included in the study and the dimensions of these wetlands are not included in Table 3.1.

The wetland sides were sloped slightly to avoid collapse. Thus, the dimensions of the wetlands were smaller at the base (approximately 10.6 m long x 1.8 m wide). Wetlands were lined with an impermeable 0.5 mm thick PVC geomembrane. Carpet was placed under the geomembrane to reduce the risk of puncture from sharp stones. The excavated soil was used to create berms around the edges of the wetlands to prevent surface water inflow or wastewater outflow. Each berm was approximately 0.2 m in height above the surrounding soil and approximately 0.5 m

in width. The wetlands were filled with topsoil taken from Shanbally, east County Cork (soil characteristics shown in Table 3.2). Following extensive soil sampling from a number of sites, soil from Shanbally was chosen due to the high percentage of silt and clay it contained to ensure that the soil used was as representative as possible of the soil that would be used in a ZDWS. At the time of soil filling, two 150 mm diameter PVC inspection pipes were placed in the centre of each wetland, 1.2 m from either end. A laser level was used to ensure that the surface of the beds were level at the end of the soil-filling process.

Table 3.1 Dimensions and surface areas of the zero-discharge wetlands

Wetland No.	Wetland type	Length (m)	Width (m)	Area (m^2)
2	[+ willow + cover]	11.5	2.4	27.6
4	[- willow + cover]	11.3	2.3	26.0
5	[- willow + cover]	11.5	2.3	26.5
7	[- willow – cover]	11.3	2.3	26.0
8	[+willow + cover]	11.5	2.5	28.8
9	[- willow + cover]	11.0	2.5	27.5
10	[+ willow + cover]	11.1	2.5	27.8
12	[+ willow + cover]	11.0	2.7	29.7

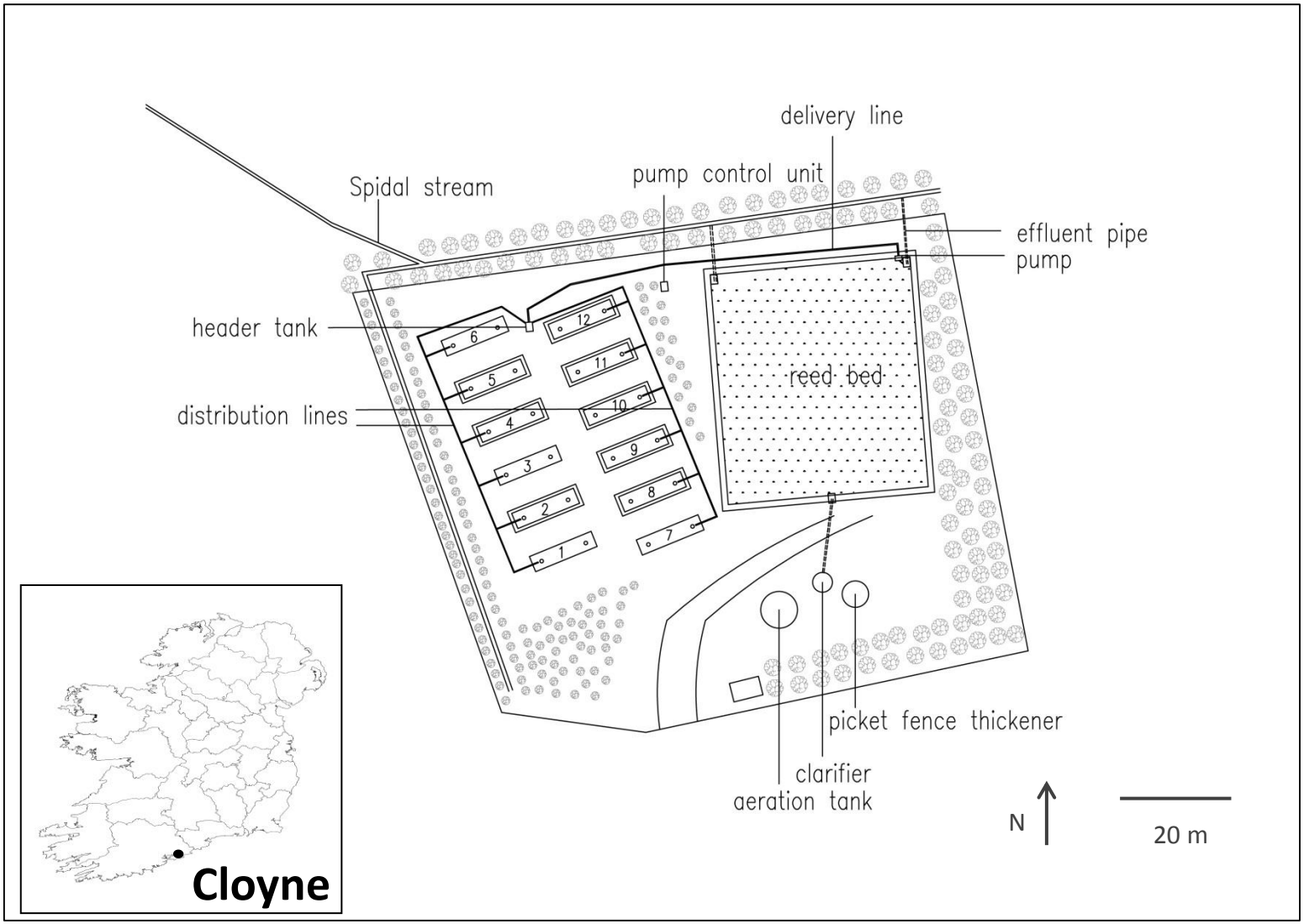


Figure 3.1 Site map of Cloyne WWTP. The ZDWS are labelled as wetlands 1-12. Also shown is the location of Cloyne on map of Ireland (inset).

3.3.1.2 Wastewater delivery system

The wastewater delivery system was installed In June 2007. A Pedrollo HF 5C 48 mm centrifugal pump (San Bonifacio, Italy) was installed at the north-east discharge point of the reed bed (Figure 3.1). Tertiary-treated effluent was pumped from this point through a 58 mm delivery line to the 1 m^3 header tank located between wetlands 7 and 12 (Figure 3.2 (A)). Wastewater pumping was controlled by a float switch located in the header tank. The header tank consisted of two 1 m^3 plastic tanks stacked one on top of the other. Each plastic tank was encased in a metal frame for support. Only the top tank contained wastewater.

Wastewater flow from the header tank to the wetlands was through gravity distribution. Wastewater entered the distribution lines through a 75 mm exit pipe, controlled by a quarter turn ball valve, and flowed to each wetland through 30 mm Tobby® No Torsion System distribution hosing (EPS, Mallow, Co. Cork, Ireland). A Kent PSM 25 mm water meter (Elster Water Metering Ltd., Luton, UK) located at the end of the distribution hosing recorded the wastewater volume input (in L) into each wetland. Wastewater was discharged onto the soil surface of each wetland from the water meter. From April to September each year the water meters remained attached to the distribution hosing. From October to March the water meter was removed from the hosing when not in use to prevent damage from frost and ice.

An automatic system for controlling wastewater inflow could not be developed. It was attempted to install an automatic ball-cock mechanism in each wetland. This was not possible due to grit from the reed bed preventing the non-return valve on the pump inflow pipe from forming a watertight seal. Air-locks then formed in the pump when the pump was automatically turned on by the float switch in the header tank. As a result of this, to prevent damage the pump could only be used under supervision and frequently needed to be manually primed before use.

(A) **(B)**



Figure 3.2 (A): wetlands shortly after installation, June 2007. The header tank is visible in the left background. (B): wetland 8, August 2008. Images: P. Whelan.

3.3.1.3 Willow planting

During August 2007 *Salix viminalis* cuttings (Alba Trees Ltd., Gladsmuir, East Lothian, Scotland) were planted in wetlands 2, 8, 10 and 12 (Figure 3.2 (B)). All trees were of the same age and clone. Very poor establishment rates occurred due to flooding of the wetlands during September 2007, leading to waterlogged soil conditions. Replanting of *Salix viminalis* trees from the same supplier took place in April 2009. The trees were planted in two pairs of double-rows in each wetland. The

within-row spacing was 0.2 m, the between-row spacing was 0.2 m and the between-double row spacing was 0.6 m. Each willow wetland contained 200 trees. In the absence of daily watering, during planting the cuttings were inoculated with DIEHARD^{TM} Transplant mycorrhizal fungi inoculum (Horticultural Alliance, Sarasota, Florida, USA) to maximise willow establishment (Appendix 1).

3.3.1.4 Polytunnel construction

During March-April 2009, polytunnels (First Tunnels, Barrowford, UK) were constructed on the four wetlands containing willow trees [+ willow + cover]. In addition, polytunnels were constructed on four other, unplanted wetlands to act as controls [- willow + cover]. One unplanted wetland was not covered by a polytunnel [- willow – cover]. Each polytunnel was approximately 12.2 m long, 3.7 m wide and 2.3 m high (exact dimensions, Figure 3.3). The foundation poles of the polytunnel were outside the berms of the wetlands. Back-filled earth along the side trenches was used to anchor the polythene cover in place. The polythene used was “Super Clear” which is a 5-year guaranteed cover. Light transmission (including UV) at installation is 95% and the polythene scatters the light as it is transmitted through the cover. This reduces the shadowing from the polytunnel frame. To allow wind to flow through the polytunnels to enhance evapotranspiration the ends of the polytunnels were left open. However, the absence of sides at the end of the polytunnels compromised the structural stability of the covers due to the passage of wind.

3.3.1.5 Polytunnel reconstruction

The current study began in October 2009. At the time, the polytunnel covers were beginning to tear due to being repeatedly stretched back and forth by the wind. Repair work was carried out from October to December 2009. However, by January 2010 it became clear that the polytunnels would have to be reconstructed due to tearing of the polythene, movement of the foundation poles in the ground and damage to the polytunnel frame. During February 2010 the polythene was cut away and all eight of the polytunnels were disassembled. A complete inventory of the conditions of all the polytunnel parts (foundation pole, side pole, hoop, ridge bar, ridge clamp, u-clip, p-clip, door rail and stabiliser) was compiled. Repairs were carried out on some damaged parts. The remainder of the damaged parts were re-ordered from the supplier.

Reconstruction of the polytunnels was done in a modified way (Figure 3.3). In April 2010, foundation pole holes (0.4 m x 0.4 m x 0.4 m) were dug for the first, fourth, sixth and ninth poles on each side. These foundation poles were placed in 10 L pots containing concrete. Once the foundation poles were set and the concrete pots placed in the holes, soil was backfilled on top of the concrete pots. The remaining foundation poles were driven in to the ground to a depth of 0.40 m. The hoops, ridge bars, end stabilisers and storm braces were then added.

During June 2010 wooden doors were installed at the ends of the polytunnels. The purpose of the doors was to reduce the amount of wind entering the polytunnel and to hold the polythene in place at either end of the tunnels. This reduced the

vibration and stretching of the polythene and kept the material taut. Door assembly was done off site in the School of BEES. Each door was 0.7 m wide and 1.95 m high. Mesh-covered panels on each door provided ventilation. The doors were hung from the polytunnel door frames and secured by burying the bottom of the door frame.

To anchor the polythene in place, trenches were dug around the sides of the polytunnels in early July 2010. This was done using a mini-digger. Trenches were 0.4 m deep and 0.5 m wide. A buffer of soil was left between the pots containing the concrete and the foundation pole, so that the polythene would not be resting up against the rough edge of the pot/concrete. Anti-hot-spot tape was applied to all areas of the polytunnel frames that would come into contact with the polythene. From late July, the polythene covers were placed on each of the frames and into the trenches. The soil was back-filled until the polythene was taut. The covers were secured by the attachment of the door frames. Views of the wetlands containing willows are shown in Figure 3.4.

To further reduce the risk of wind damage, windbreaks were constructed at the western ends of each of the polytunnels in November 2010. Each windbreak consisted of two 2.5 m timber posts, and two 6 cm x 9 cm wooden beams to act as crossbars. The timber posts were placed 2 m apart and centred 2 m from the western door. Windbreak mesh was then attached to the frame.

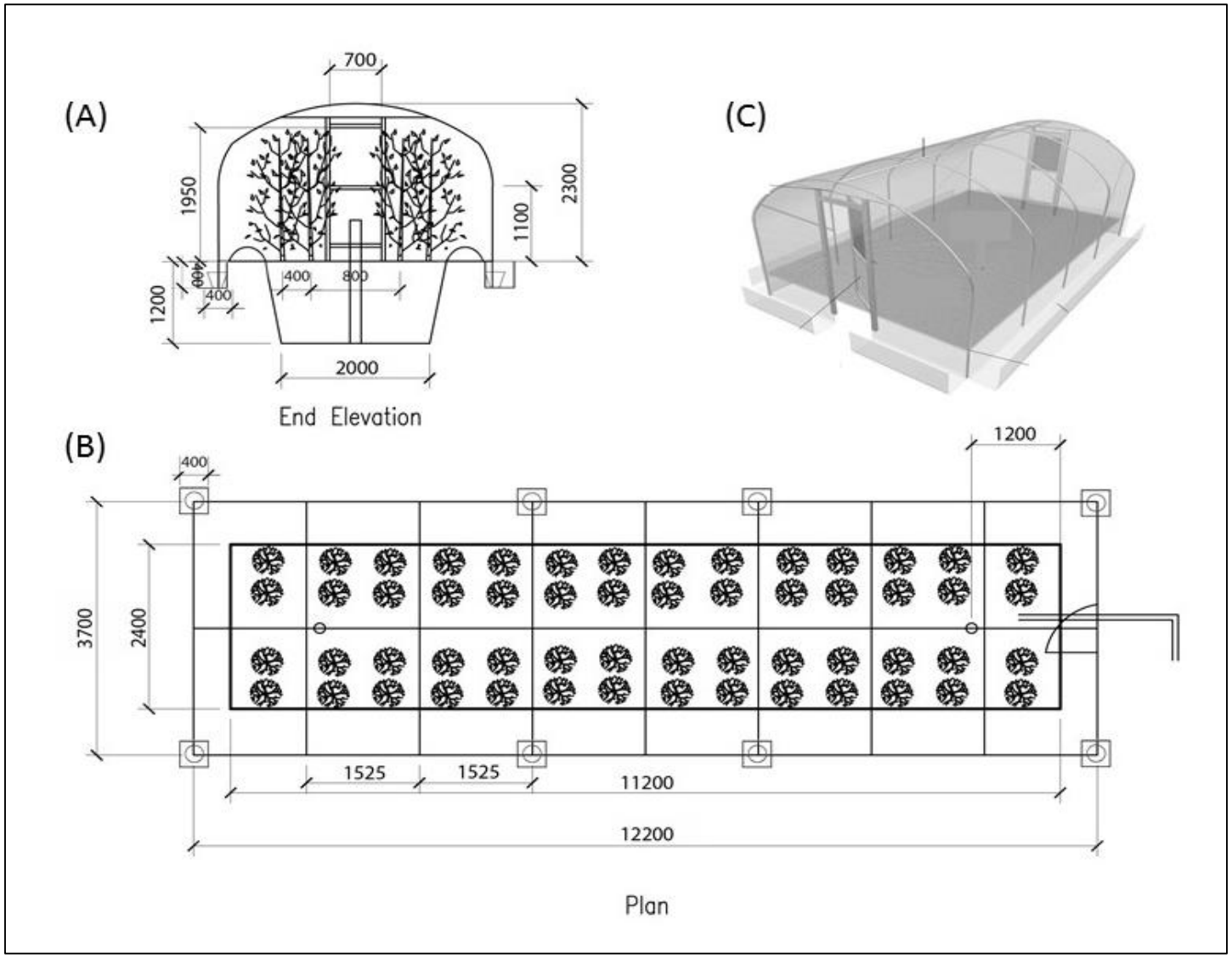


Figure 3.3 (A) End elevation and (B) plan of ZDWS. All dimensions in mm. (C) 3D visualisation of polytunnel.

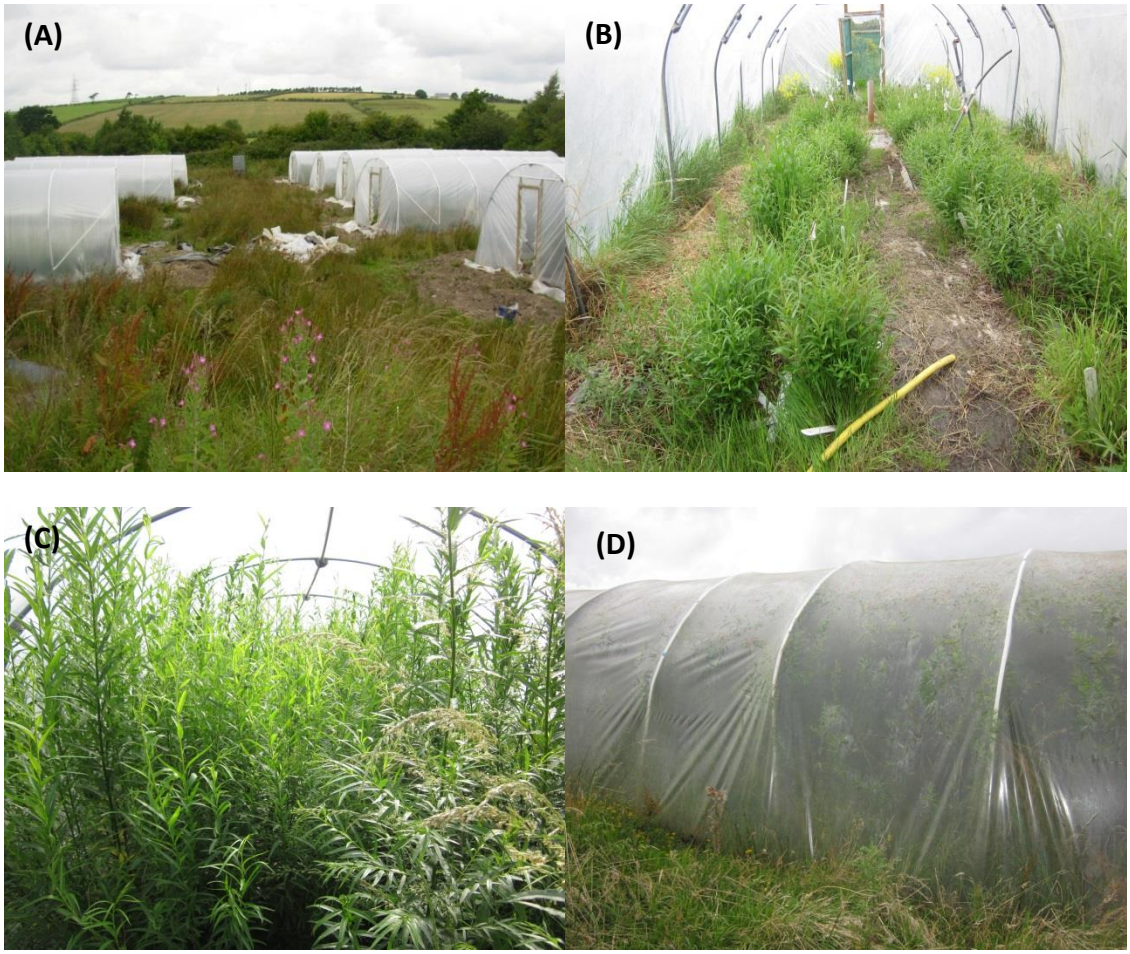


Figure 3.4 Views of zero-discharge willow wetlands

(A): View looking north, July 2010. (B): wetland 10, May 2012. (C): wetland 10, June 2012. (D): wetland 10, August 2012.

3.3.1.6 Soil analysis

Soil pH was measured using a 1:5 ratio of air-dried soil to water with an Orion 3 soil pH meter (Thermo Electron Corporation, Singapore)(Rowell, 1994). Soil electrical conductivity was measured using a 1:5 ratio of air-dried soil to deionised water with a WTW electrical conductivity probe (WTW, Weilheim, Germany)(Rowell, 1994). Ten grams of air-dried soil was added to 50 ml of deionised water and shaken for one hour before settling for 30 minutes and measurement. Organic matter content was measured using the loss-on-ignition (LOI)) method which measures the

gravimetric loss of organic matter from oven-dried soil at 500 °C in a furnace (Rowell, 1994).

Soil texture analysis was carried out using the laser diffraction technique. Soil samples were wet-sieved through a 2 mm sieve and then air dried. Three grams of this fraction were placed into a 250 mL beaker. Twenty mL of 30% H_2O_2 were added to remove the organic matter (Carter, 1993). Further H_2O_2 was added and the mixture was slowly heated using a hot plate to approximately 90°C to complete the removal of the organic matter (until frothing ceased). The sample was then allowed to cool. The peroxide-treated soil was then shaken with a dispersal agent of 10% sodium hexametaphosphate on a mechanical shaker for 24 hours to destroy aggregates (Rowell, 1994). The dispersed samples were analysed using a Malvern Mastersizer 2000 (Malvern, Worcestershire, UK) by means of liquid dispersion and sonication (Table 3.2).

Table 3.2 Soil analysis of the soil used in the ZDWS

Parameter	Value
pH	8.4
Electrical conductivity$_{1:5}$ (µS cm^{-1})	71.2
Organic matter content (% LOI)	6.1
Sand (%)	54.8
Silt (%)	36.7
Clay (%)	8.5

3.3.2 Meteorological data

Monthly rainfall data, temperature data and mean global solar radiation (GSR) data from the Met Éireann weather station in Roche's Point (approximately 10 km to the south-west of the site, elevation = sea level) were used. Temperature and relative

humidity were recorded on site using HOBO® Pro v2 data loggers (Onset Computer Corporation, Bourne, Massachusetts, USA). Loggers were located in wetland 10 [+ willow + cover], wetland 4 [- willow + cover] and wetland 7 [- willow – cover]. Monthly total sunshine hours data were taken from the Met Éireann station in Ballincurrig (approximately 15 km north-west of the site, elevation = 73 m). Monthly reference evapotranspiration data were taken from the Met Éireann station at Cork Airport (approximately 20 km west of the site, elevation = 143 m). Reference evapotranspiration at Cork Airport station is calculated by Met Éireann using the Penman-Monteith formula following the methods of Allen *et al.* (1998).

3.3.3 Wetland wastewater irrigation and evapotranspiration measurement

Wastewater evapotranspiration was calculated following the working principle of the Danish ZDWS; on an annual basis evapotranspiration is equal to the amount of wastewater inputted to each ZDWS:

$$ET = Q \qquad \textbf{Equation 2.5}$$

To determine how much wastewater to input to the wetlands, a system was developed whereby the wetlands would be filled to the same point on each visit to the field site. On the inside of the wetland inspection pipes, a level was marked which corresponded to the soil surface of the wetland. Wastewater was inputted until the wastewater level inside the inspection pipe furthest from the water meter had reached this set mark. The water meter was then closed preventing more wastewater from entering the wetland. The amount inputted would then be equal to the amount lost through evapotranspiration over the time period from the last wastewater application. Wastewater use was recorded each month for the years

2012-2013, except during February 2012 due to problems with the pump distribution system. Wastewater irrigation amounts were summed on a monthly basis and expressed as mm day^{-1} for each month.

For wetland 7, in times of very heavy rainfall the berms were breached leading to water loss from the wetland. This water loss was not quantifiable. It was not possible to assume that all of the rainwater that fell was retained in the wetland, as this would have given spuriously high evapotranspiration values for this wetland (>1400 mm annually). Thus, for wetland 7 values are expressed only as ET with respect to wastewater input.

Crop factor values were calculated as:

$$k_c = ET/ET_O \quad \textbf{Equation 2.4}$$

and thus strictly represent the ratio between wetland type ET and ET_O, rather than the ratio between willow crop ET and ET_O.

3.3.4 Tree measurements, harvesting and maintenance

Willow measurements were taken each year before coppicing during January - February for 2010 to 2013. A census of the tree population in each wetland was taken. A subsample of twenty trees per wetland was taken annually and the height, diameter at 0.30 m and number of primary shoots per willow stool were counted. Willows were coppiced by hand at approximately 0.15 m above the soil surface. Fresh weight of coppiced material was measured on site. A subsample of trees (10% of the total willow fresh weight per wetland) was taken off site and dried in an oven

at 70°C until constant weight. The dry weight of the coppiced material in each wetland was then calculated from the percentage moisture lost by the subsample.

At the start of the experimental period the weeds in all of the wetlands were sprayed with glyphosate and a woven geotextile was laid down to prevent weed regrowth. During the summer of 2011 a caterpillar infestation occurred and all willow trees were sprayed with *Bacillus thuringiensis* (Fruit Hill Farm Ltd., Bantry, Co. Cork). During the summer of 2012, an aphid infestation occurred in wetland 8. Decis® (Bayer AG, Leverkusen, Germany) was sprayed on affected trees to eradicate the aphid infestation.

3.3.5 Wastewater quality monitoring and nutrient loading

Quality data of the influent wastewater were supplied by EPS Group. Sampling was carried out at the outflow pipe of the reed bed. Weekly testing was carried out for pH, COD, TSS, TN and TP by EPS. BOD_5 was measured externally by an accredited laboratory, Exova Ltd. Once per month a full suite of water quality analysis was carried out by Exova Ltd. Nutrient loading was calculated as the product of the volume of wastewater irrigated on to each wetland and the nutrient concentration in the wastewater for the closest available date.

3.3.6 Statistical analysis

All data approximated to normality (with/without transformation). Differences in meteorological conditions and reference evapotranspiration data (log_{10} transformed) between 2012 and 2013 (Met Éireann data) were analysed by the paired t-test. Temperature (monthly maximum temperature data were normalised by square root transformation) and humidity data recorded by the HOBO loggers in

the different wetland types were analysed by one-way ANOVA. Evapotranspiration data (log_{10} transformed) were subjected to two-way ANOVA to statistically test the effects of wetland type and year. Differences in wastewater quality between 2012 and 2013 were analysed by paired t-tests. Wastewater quality data were square root transformed where necessary. Willow growth parameters were transformed where necessary and analysed using one-way ANOVA to test the effect of year. Post-hoc tests were carried out using the Tukey test. Descriptive statistics are presented as non-transformed values. Statistical analyses were carried out using SPSSv20 (IBM, New York, USA).

3.4 Results

3.4.1 Meteorological conditions

Annual rainfall totals for 2012 and 2013 were not significantly different (paired t = 0.275, 11df; p > 0.05), with rainfall of 944 mm in 2012 and 1,006 mm in 2013 (Figure 3.5). June 2012 was the wettest month during the two-year experimental period with 185 mm of rainfall, while September 2012 was the driest month (13 mm). The growing season of 2013 (April to September) was drier than 2012, with 455 mm falling during April to September 2013, compared to 706 mm during the same period in 2012, though the difference was not significant (paired t = 1.17, 5 df; p > 0.05).

There was no significant difference in average temperature (paired t = 0.02, 11 df; p > 0.05) between 2012 and 2013, with an annual average temperature of 10.6 °C in both years. The coldest month in 2012 was December (mean temperature of 7.3 °C), while in 2013, March was the coldest month (5.1 °C). August was the warmest

month in 2012 (mean temperature of 15.8°C), while July was the warmest month in 2013 (17.4°C).

The total annual reference evapotranspiration in 2013 (512 mm) was higher than that for 2012 (344 mm) but this difference was not significantly different (paired t = 2.02, 11 df; p > 0.05)(Figure 3.5). Reference evapotranspiration during the growing season of 2013 was 416 mm, which was significantly greater than reference evapotranspiration during the 2012 growing season (272 mm)(paired t = 3.59, 5 df; p < 0.05). December of both years saw the lowest monthly reference evapotranspiration of 9 mm.

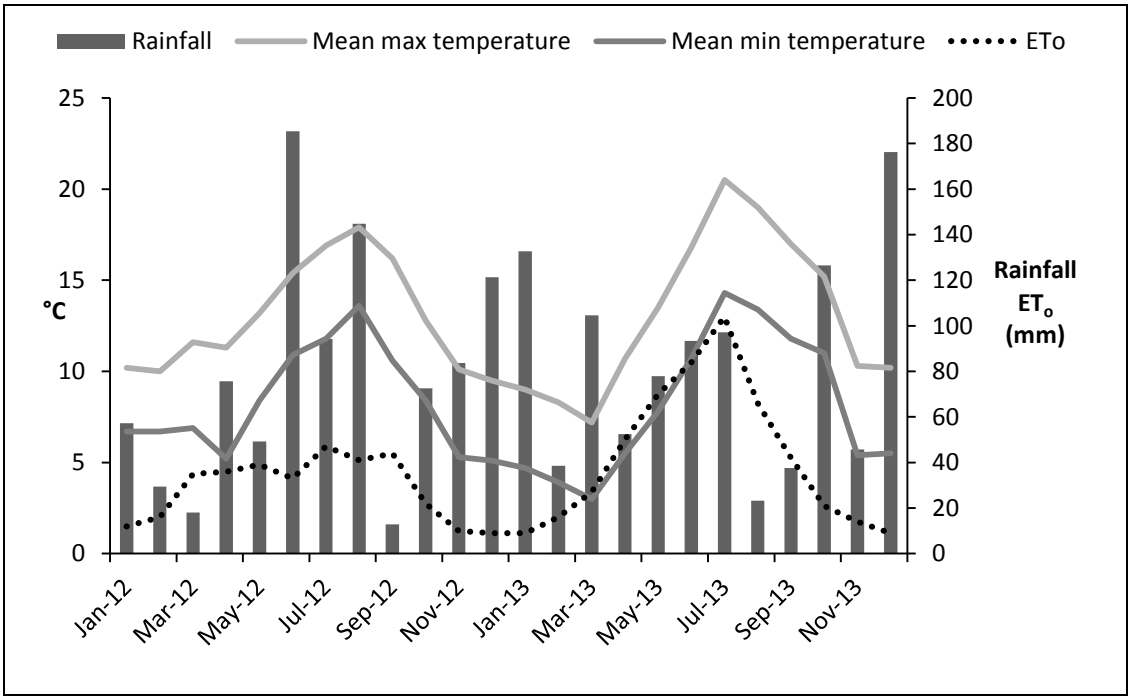


Figure 3.5 Monthly rainfall, mean monthly maximum temperature, mean monthly minimum temperature and reference evapotranspiration from 2012 to 2013.

The total amount of global solar radiation received in 2013 was 3,904 MJ m^{-2} (Figure 3.6). This was higher than in 2012 (3,364 MJ m^{-2}) but the difference was not significant (paired t = 1.77, 11 df; p > 0.05). The total amount of global solar radiation received during the growing season of 2013 was higher than that received during 2012 with the difference approaching significance (paired t = 2.15, 5 df; p = 0.054). Similarly, the 2013 growing season had more sunshine hours than in 2012, though the difference was not significant. For each month from April to August 2013, more than 165 hours of sunshine occurred. In comparison, in 2012, more than 165 hours of sunshine occurred only during April. June 2012 was very dull (86 hours) which was only 45% of the sunshine hours in June 2013 (191 hours). The highest monthly number of sunshine hours recorded was 260 hours during July 2013.

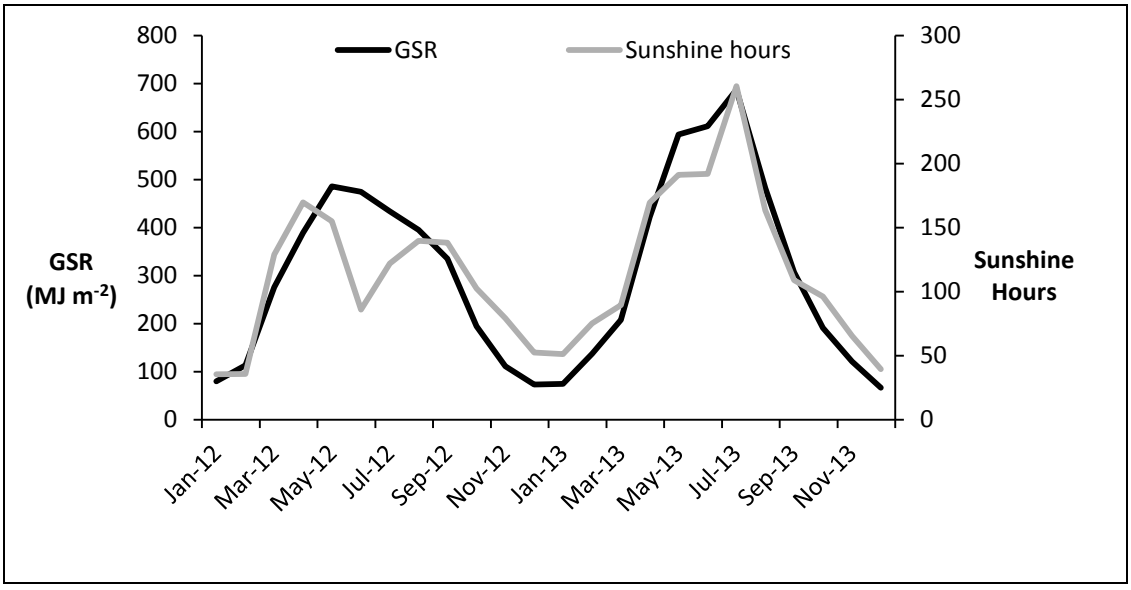


Figure 3.6 Monthly global solar radiation and monthly number of sunshine hours 2012-2013.

Monthly average temperatures recorded using the HOBO data loggers were generally higher inside the polytunnels than outside (Figure 3.7 A). There was no

significant difference in mean annual average temperature ($F(2,33) = 1.69$; $p > 0.05$). Monthly maximum temperature was generally higher in the [- willow + cover] wetlands and the [+ willow + cover] wetlands, compared to the [- willow – cover] wetland (Figure 3.7 B). Mean annual maximum temperature was significantly higher in the [- willow + cover] wetlands than the [- willow – cover] wetland ($F(2,33) = 3.27$; $p = 0.05$). Monthly minimum temperatures were similar (Figure 3.7 C) and there was no significant difference in mean annual minimum temperature ($F(2,33) = 0.00$; $p > 0.05$). Relative humidity in each of the three settings varied from 70% to 95% (Figure 3.7 D) and there was no significant difference in mean annual relative humidity ($F(2,33) = 0.35$; $p > 0.05$).

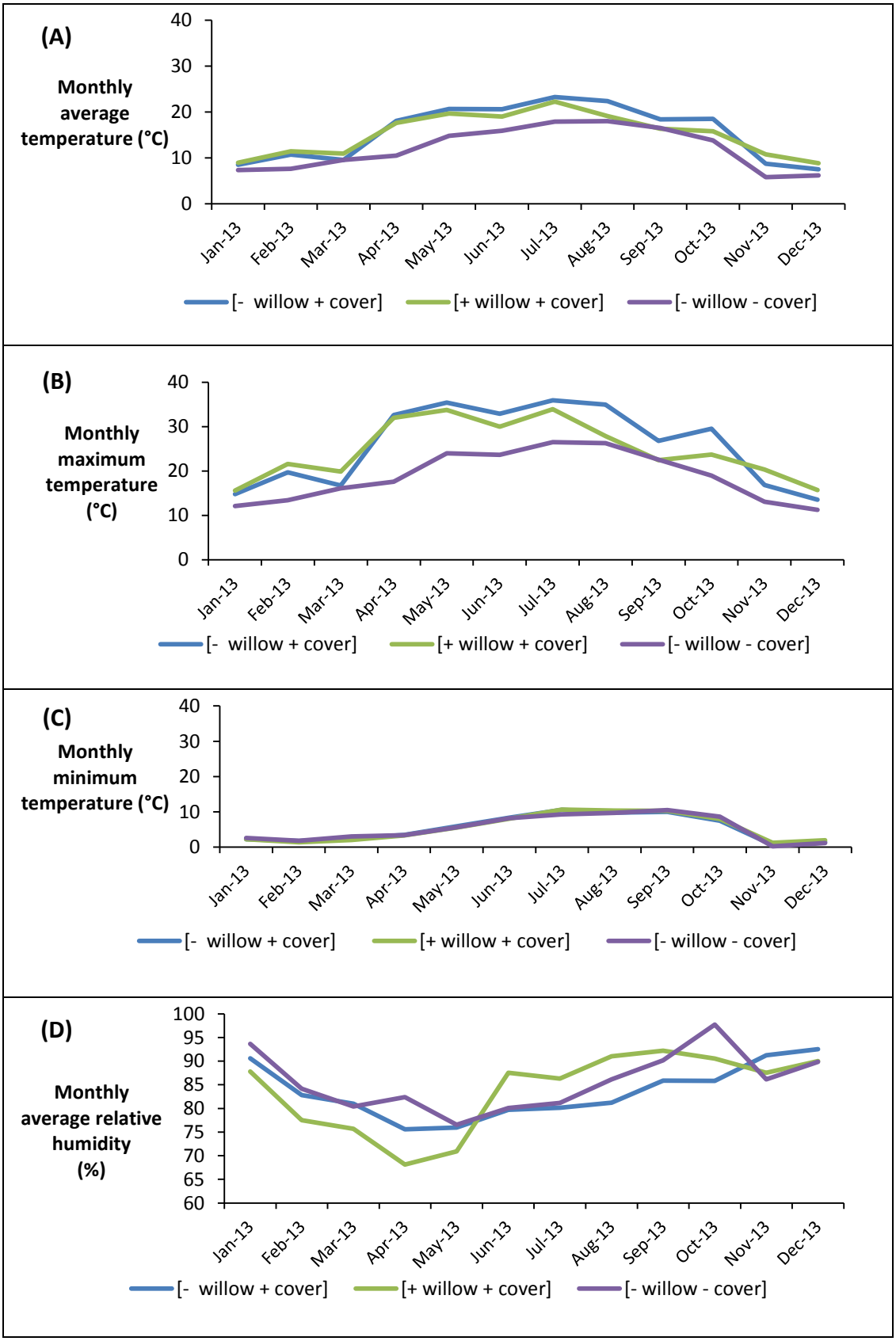


Figure 3.7 Meteorological data recorded on site: January 2013 – December 2013. (A) Mean monthly average temperature; (B) mean monthly maximum temperature; (C) mean monthly minimum temperature; (D) mean monthly average relative humidity.

3.4.2 Evapotranspiration

The average daily ET for each month during the study period varied on a seasonal cycle (Figure 3.8). In both years, ET was highest during the growing season (April – September) with lower values during the dormant season (October – March). During 2012, average daily ET values peaked during September for the [+ willow + cover] wetlands (3.0 mm day^{-1}), and May for the [– willow + cover] treatments (2.6 mm day^{-1}). Peak values increased in 2013, reaching 4.8 mm day^{-1} in August [+ willow + cover] and 3.7 mm day^{-1} in July and August [- willow + cover]. For the [– willow – cover] wetland, peak values were much lower even during summer periods due to incident rainfall. During the period June – August 2012, no wastewater was loaded onto the [– willow – cover] wetland as the water level was at the soil surface. In 2012 the highest monthly ET for the [– willow – cover] wetland occurred during May (2.1 mm day^{-1}), while in 2013 the highest average monthly ET occurred during July (2.9 mm day^{-1}).

During the dormant season, ET values were lower than during the growing season. Values showing zero in Figure 3.8 indicate that no wastewater was loaded into the wetlands during these months (except during February 2012 when the pump was broken). For the [+ willow + cover] wetlands the lowest average daily ET that occurred was 0.7 mm day^{-1} during December in both years. The lowest ET values for the [– willow + cover] wetlands occurred in January 2012 and February 2013 during which months no wastewater was inputted. Of the months where wastewater was inputted, the lowest ET value during 2012 occurred in December (0.5 mm day^{-1}) and the lowest ET value in 2013 occurred during January (0.3 mm day^{-1}). For the [–

willow – cover] wetland, the lowest non-zero values occurred during March in both years (1.0 mm day^{-1} in 2012 and 0.4 mm day^{-1} in 2013).

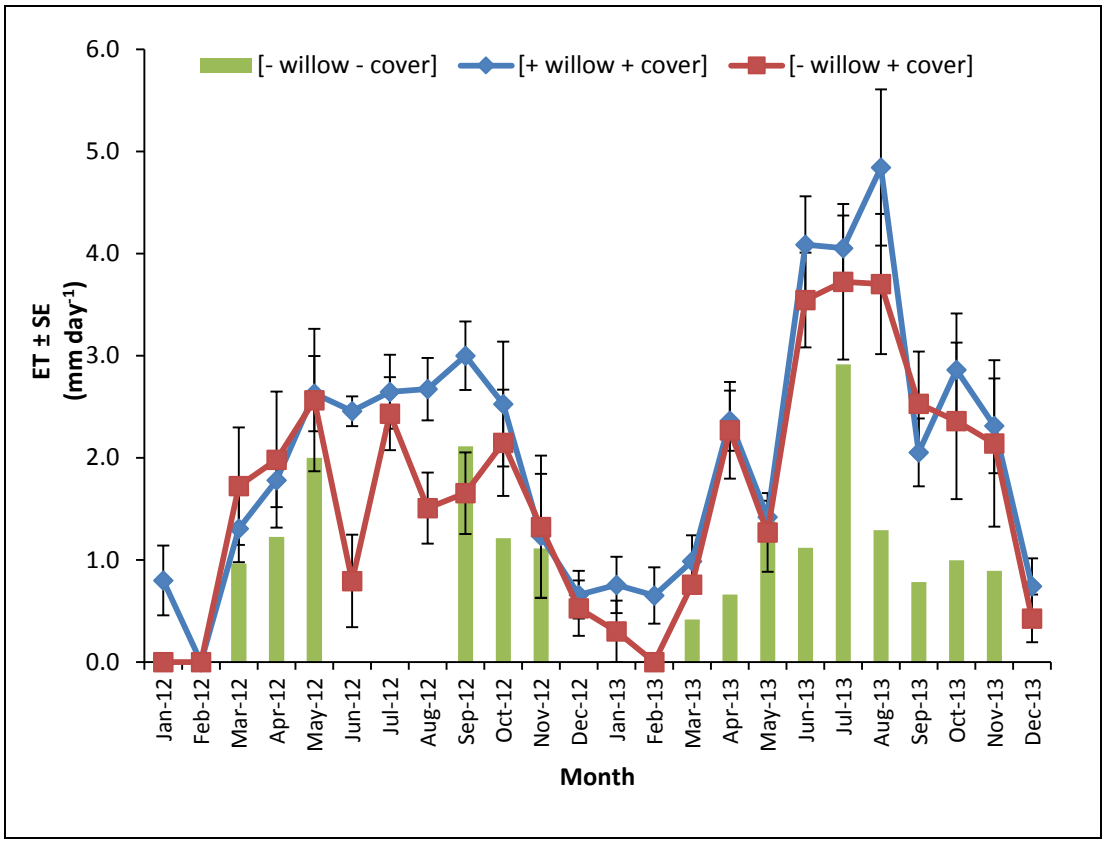
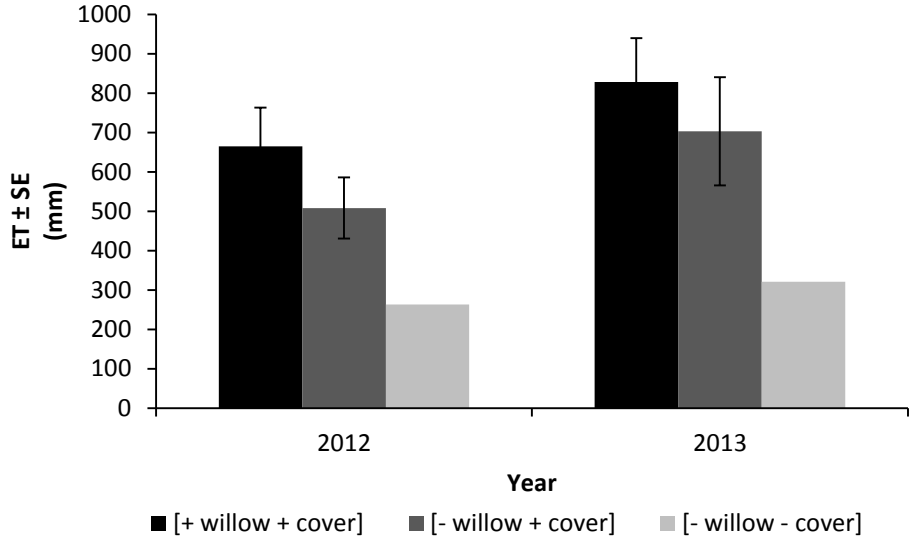


Figure 3.8 Average daily ET (± SE where appropriate) for the three wetland types: [+ willow + cover], [- willow + cover] and [- willow – cover] during the 24 month experimental period.

The [+ willow + cover] wetlands exhibited the highest evapotranspiration in both 2012 and 2013 (Figure 3.9). In 2012, average evapotranspiration from the [+ willow + cover] wetlands was 665 mm which rose to an average of 828 mm in 2013. The [– willow + cover] exhibited lower evapotranspiration rates in both years; 508 mm in 2012 and 703 mm in 2013. The [– willow – cover] wetland showed the lowest evapotranspiration in both years, with 263 mm in 2012 and 321 mm in 2013. Wetland type had a significant effect on evapotranspiration, though the effect of year and interaction were not significant. Annual evapotranspiration of the [+

willow + cover] wetlands over the two years was significantly larger than the [- willow – cover] wetland. The annual evapotranspiration total of the [– willow + cover] wetlands did not differ significantly from either of the other two wetland types over the two years.



ANOVA	df	F	p
Wetland	**2**	**7.05**	**<0.05**
Year	1	1.77	>0.05
Wetland x Year	2	0.04	>0.05

Figure 3.9 Total annual ET (± SE where appropriate) for the three wetland types: [+ willow + cover], [- willow + cover] and [- willow – cover] during the 24 month experimental period.

3.4.3 Crop factors

For all of the wetland types, the highest k_c values were recorded in November of both years: 3.7 in 2012 and 5.0 in 2013 for [+ willow + cover], 4.0 in 2012 and 4.6 in 2013 for [– willow + cover], and 3.3 in 2012 and 1.9 in 2013 for the [– willow – cover] wetland (Figure 3.10). Crop factors for the [– willow – cover] wetland were the lowest in 23 of the 24 months studied, the exception being during September 2012, when the k_c value of 1.4 was slightly higher than the average k_c value of 1.1 recorded for the [– willow + cover] wetlands. Any months showing a k_c value of zero

indicates that no wastewater was inputted during these months (except February 2012 as stated previously). In 2012 the lowest average k_c value for the [+ willow + cover] wetlands was 1.2 recorded in March. During 2013 the lowest k_c value of 0.6 was recorded in May. For the [– willow + cover] wetlands the lowest values were June 2012 (0.7) and February 2013 (0.6). During nine of the 24 months studied no wastewater was inputted to the [– willow – cover] wetland resulting in k_c values of zero. The lowest k_c values for months where wastewater was inputted were March 2012 (0.9) and April 2013 (0.4).

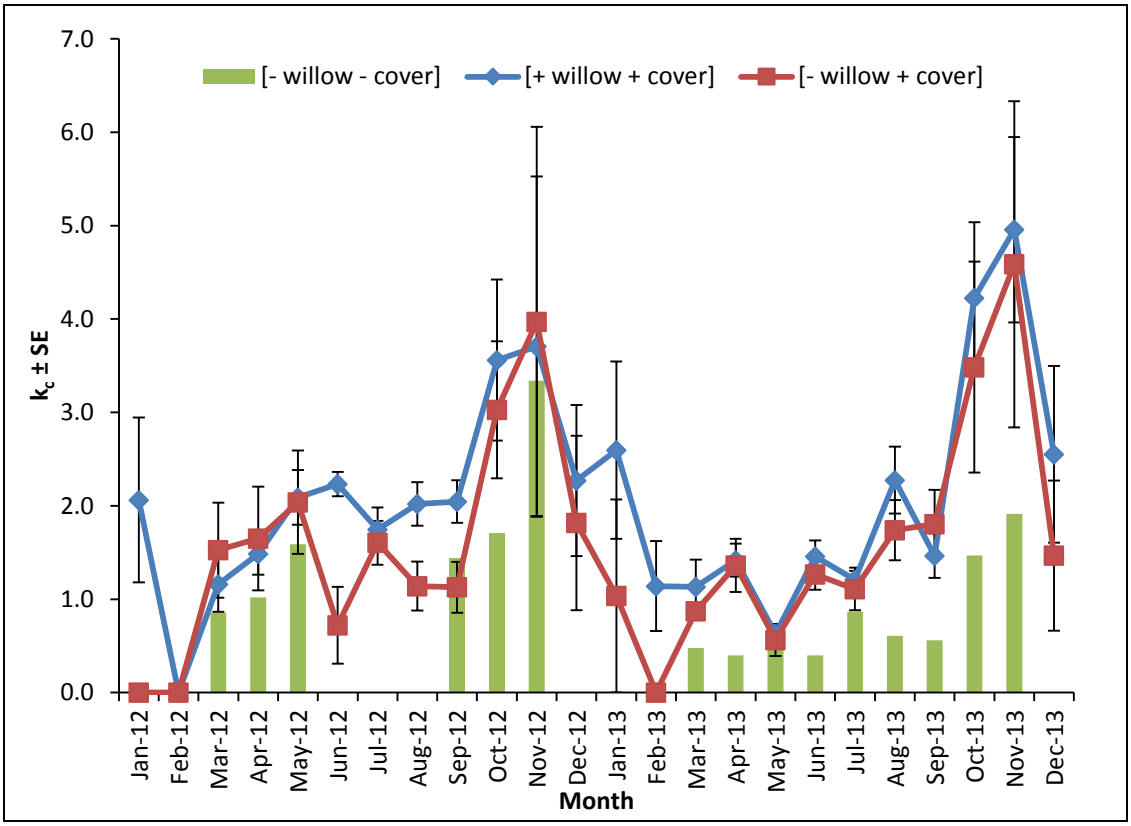


Figure 3.10 Average monthly k_c values (± SE where appropriate) for [+ willow + cover], [- willow + cover] and [- willow – cover] during the 24 month experimental period.

Mean annual crop factors for the [+ willow + cover] wetlands were the highest of the three wetland types in both years (Table 3.3) ranging from 2.0 in 2012 to 2.1 in

2013. Mean annual crop factors were lowest for the [- willow – cover] wetland; 0.8 in 2012 and 0.6 in 2013.

Table 3.3 Mean ± SE annual k_c values for 2012 and 2013

Wetland type	2012	2013
[+ willow + cover]	2.0 ± 0.28	2.1 ± 0.38
[- willow + cover]	1.6 ± 0.33	1.6 ± 0.36
[- willow – cover]	0.8 ± 0.3	0.6 ± 0.17

3.4.4 Willow growth and survival

While the years preceding 2012 do not form part of the evapotranspiration monitoring, the results of the biomass harvests for these years have been included (Figure 3.11). Biomass production from 2007 to 2010 was low (2.7 odt ha^{-1} (oven dry tonnes per hectare)). The influence of the first coppice during the winter of 2010-2011 can be seen on the yield produced in 2011. Yield increased to 11.5 odt ha^{-1}, however there was no significant difference in willow yield during any of the four years ($F(3.12) = 3.32$; $p > 0.05$). In 2012, yield decreased slightly to 9.5 odt ha^{-1} and again decreased in 2013 to 8.0 odt ha^{-1}.

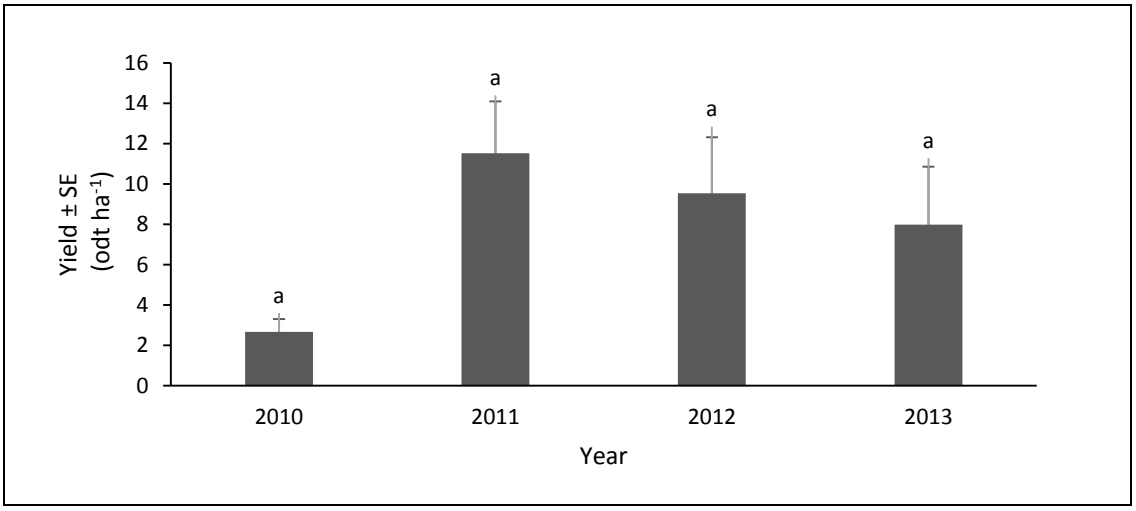


Figure 3.11 Mean biomass ± SE yield from 2010 to 2013 for the [+ willow + cover] wetlands. Any samples which share a common letter are not significantly different using the Tukey test (p > 0.05).

Shoot height of the [+ willow + cover] ZDWS varied from 182.1 cm in 2011 to 190.2 cm in 2012 (Table 3.4). There were no significant differences in height between years ($F(2,9) = 0.23$: $p > 0.05$). While leading shoot diameter decreased each year, from 9.9 mm in 2011 to 9.0 mm in 2012, there were no significant differences due to year ($F,(2,9) = 0.51$; $p > 0.05$). There was a significant difference in primary shoot number, with trees in 2011 having a significantly lower shoot number than in 2012 and 2013 ($F(2,9) = 14.18$; $p < 0.01$). There was a significant difference in percentage survival ($F(2,9) = 0.744$; $p < 0.05$). Survival in 2011 (100%) was significantly higher than in 2013 (80.7%). Survival in 2012 (85.5%) was not significantly different from that in either 2011 or 2013.

Table 3.4 Mean ± SE willow growth parameters and survival for the growing seasons of 2011-2013 for the [+ willow + cover] ZDWS. Any samples which share a common letter are not significantly different using the Tukey test (p > 0.05).

2011	2012	2013
Leading shoot height (cm)		
182.1 ± 9.54 a	193.1 ± 11.67 a	190.2 ± 13.6 a
Leading shoot diameter$_{0.3}$ (mm)		
9.9 ± 0.32 a	9.8 ± 0.74 a	9.0 ± 0.78 a
Primary shoot number per tree		
5.6 ± 0.07 a	18.7 ± 3.51 b	18.6 ± 2.83 b
Percentage survival (2011 = 100%)		
100% a	85.5% ± 3.91 ab	80.7% ± 5.05 b

3.4.5 Wastewater quality and nutrient loading

The pH of the wastewater entering the wetlands was similar in both years (paired t = 0.60, 11 df; p > 0.05) (Table 3.5). There were no significant differences in COD (paired t = 0.93, 11 df; p > 0.05), BOD (paired t = 1.18, 11 df; p > 0.05) and TSS (paired t = 0.33, 11 df; p > 0.05) between 2012 and 2013. TN showed the largest difference between years, but the difference was not significant (paired t = 2.149, 11 df; p > 0.05). Over the course of 2012 the average TN concentration was 15.3 mg L^{-1} which was lower, though not significantly so, than the value of 25.8 mg L^{-1} for 2013. In 2013 the average TP concentration of 2.6 mg L^{-1} was slightly higher than the corresponding concentration for 2012 of 2.2 mg L^{-1}, but this difference was not significant (paired t = 0.82, 11 df; p = 0.43).

The TN concentration of the wastewater showed considerable variation across the 24-month study period, ranging from 3.8 to 55.0 mg L^{-1} (Figure 3.12). The TN concentration was lowest from September 2012 to January 2013, varying from approximately 4.1 to 8.6 mg L^{-1}. In the second half of 2013, the TN concentration

was generally higher, ranging from 16.8 to 55.0 mg L^{-1}. The TP concentration in the wastewater during 2012 varied from 1.2 to 3.8 mg L^{-1}. In 2013, TP concentrations showed a wider range, varying from 0.6 to 4.65 mg L^{-1}.

Table 3.5 Mean ±SE quality parameters of input wastewater 2012-2013. Any two samples which share a common letter are not significantly different using the paired t-test (p > 0.05).

Parameter	2012	2013
pH	7.9 ± 0.2 a	7.9 ± 0.3 a
COD (mg L^{-1})	23.1 ± 10.3 a	26.0 ± 12.4 a
BOD (mg L^{-1})	4.4 ± 2.3 a	6.4 ± 10.7 a
TSS (mg L^{-1})	3.5 ± 2.0 a	3.6 ± 2.9 a
TN (mg L^{-1})	15.3 ± 8.5 a	25.8 ± 12.7 a
TP (mg L^{-1})	2.2 ± 0.7 a	2.6 ± 1.1 a

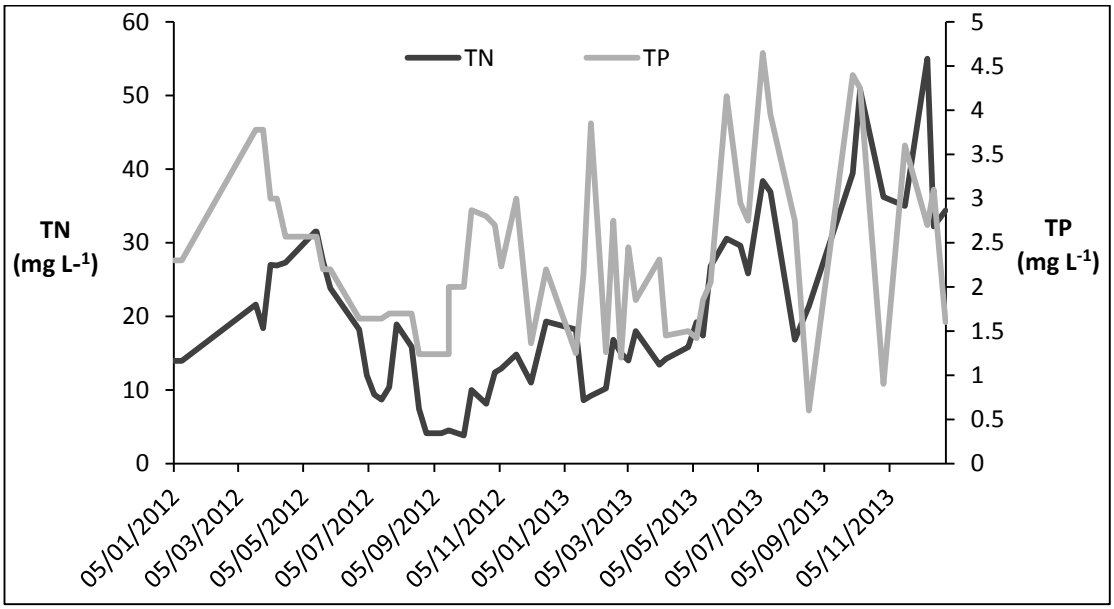


Figure 3.12 TN and TP concentration in the irrigation wastewater 2012-2013.

There was a significant increase in nitrogen loading for the [+ willow + cover] wetlands from 2012 to 2013 ($t = 4.75$, 6 df; $p < 0.01$). Annual nitrogen loading

increased from 104.5 kg N ha^{-1} in 2012 to 259.3 kg N ha^{-1} in 2013. There was also a significant increase in phosphorus loading for the [+ willow + cover] wetlands from 2012 to 2013 ($t = 2.69$, 6 df; $p < 0.05$). Annual phosphorus loading increased from 14.5 kg N ha^{-1} in 2012 to 24.4 kg P ha^{-1} in 2013.

3.5 Discussion

The use of ZDWS as a means of domestic wastewater disposal has previously been demonstrated in Denmark (Gregersen and Brix, 2001). Curneen and Gill (2015) reported that due to the high rainfall rates in Ireland the achievement of true "zero-discharge" operation is not possible and overflow from the ZDWS will occur during winter months. This study has shown that a novel polytunnel-covered ZDWS has potential for use in the Irish setting.

Evapotranspiration

The average annual evapotranspiration results for the [+ willow + cover] wetlands of 665 mm in 2012 and 828 mm in 2013 were similar to the annual ZDWS evapotranspiration (445 – 928 mm) recorded by Curneen and Gill (2015) for full-scale ZDWS in Co. Wexford, Ireland. Wetland 10 was the best performing individual [+ willow + cover] wetland and achieved an annual evapotranspiration rate of 951 mm in 2012 and 1,048 mm in 2013. This evapotranspiration rate was higher than the best performing ZDWS in the Curneen and Gill (2015) study, but lower than the annual total of 1,135 mm calculated from modelled data in the same study. However, these modelled data assumed no water-limited conditions in the willow systems during the summer months. Due to the manual irrigation mechanism used in this study, and depending on the frequency of visits during the summer months,

it is likely that water-limited conditions did occur occasionally, which would have lowered the summer evapotranspiration rates.

The annual [+ willow + cover] evapotranspiration rate compared favourably with the results of Curneen and Gill (2014) where annual evapotranspiration in years two and three for willows receiving secondary treated effluent in Ireland were 508 mm and 617 mm, respectively. Total ET in this study was also similar to the annual ET of 870 mm recorded by Martin and Stephens (2006) using willow lysimeters in the UK. As expected, daily evapotranspiration rates peaked in both years during late summer. Daily ET values during the growing season of 2.0 to 4.6 mm day^{-1} were lower than the daily ET values recorded by Curneen and Gill (2015) where maximum values were as high as 6 – 7 mm day^{-1} during June – August.

Compared to results from Denmark, evapotranspiration rates recorded in this study were lower than the annual evapotranspiration recorded by Gregersen and Brix (2001) in ZDWS in Denmark (900 – 1,200 mm in the first year and 1,400 – 2,000 mm in the second year) and also the annual evapotranspiration of 1,300 mm recorded by Ministry of Environment and Energy (2001). The lower total evapotranspiration occurring for these systems in Ireland has been attributed to higher relative humidity in Ireland than Denmark (Gill *et al*., 2014). In addition, the ZDWS were not situated broadside to the prevailing wind in Cloyne as would normally be the case in Denmark to maximise evapotranspiration (Brix and Arias, 2011). While the polytunnels were arranged so that the prevailing wind from the south-west would enter, the presence of the windbreak mesh and the small size of the door opening limited air movement within the polytunnel.

The increase in total evapotranspiration of the [+ willow + cover] wetlands from 2012 to 2013 was likely to have been due to the increased reference evapotranspiration during the growing season in 2013, as increasing temperature is known to increase evapotranspiration (Allen *et al*., 1998; Curneen and Gill, 2014). The reference evapotranspiration during the growing season of 2013 was significantly higher than that in the same period in 2012, with an additional 144 mm occurring. This figure is very close to the average increase in actual evapotranspiration of 163 mm which took place in 2013 compared to 2012 for the [+ willow + cover] wetlands. The influence of the higher reference evapotranspiration in 2013 is also reflected in the increased evaporation from the [– willow + cover] wetlands and the increased evaporation from the [– willow – cover] wetland in 2013.

The total evaporation levels from the [– willow + cover] wetlands were much higher than values of approximately 200 mm evaporation during the summer recorded by Białowiec *et al.* (2007a) using unplanted lysimeters under a roofed system. The ratio of evapotranspiration/evaporation i.e. [+ willow + cover]/[- willow + cover] was 1.31 in 2012 and 1.18 in 2013. These ratios are similar to the lowest ratio (1.28) found by Białowiec *et al.* (2007b) for the irrigation of willows and unplanted soil with landfill leachate. In general, the ratio of evapotranspiration/evaporation is much higher and can be as high as 14 for willows and soil irrigated with dilute landfill leachate (Białowiec *et al*., 2003).

Polytunnels in hot climates reduce evaporation due to lower incident solar radiation, lower wind speed and higher relative humidity (Bello, 1982; Fernandes *et*

al., 2003; Orgaz *et al*., 2005; Eamus *et al*., 2006), but this did not occur in this study under more temperate conditions. Instead, the presence of the polytunnels raised evaporation above reference evapotranspiration. In this study the wastewater was applied across the surface which is not in line with the Danish guidelines. One potential benefit of this was that the tertiary wastewater was available at the top of the soil column, rather than the bottom, which may have increased evaporation rates in the tree-less systems as wastewater did not need to move up through the soil column by capillary action. Solar radiation exerts strong control over evaporation (Allen *et al*., 1998; Fernández *et al*., 2010) and in this study it appears that evaporation was maximised from damp soil due to surface irrigation.

In addition, the low evapotranspiration/evaporation ratio may have indicated that either the willow transpiration rate was limited, perhaps due to low wind speed levels within the polytunnel, or that there may have been occasional wastewater loss from the [– willow + cover] wetlands. If occasional wastewater loss from the system did in fact occur, it was likely not to a large extent as the overall crop factor for the [– willow + cover wetlands] was 1.6 in both 2012 and 2013. This value is similar to the annual crop factor values of 1.4 or less used for reedbeds in Ireland and the UK (Peacock and Hess, 2004; O'Luanaigh, 2007; O'Luanaigh *et al*., 2010).

Total wastewater application to the [– willow – cover] wetland was much lower than the annual reference evapotranspiration in both years. This indicates some entry of surface water across the berms as was observed at site visits during time of heavy rainfall. The berms around this wetland were somewhat lower compared to the berms surrounding the other wetlands, and the wetland was also located near

the base of a moderate upslope. The crop factor values for this wetland were 0.8 in 2012 and 0.6 in 2013, both of which are below what would be expected for CWs in Ireland and the UK (Peacock and Hess, 2004; O'Luanaigh, 2007; O'Luanaigh *et al*., 2010). No wastewater input occurred for the [– willow – cover] wetland during the summer of 2012. This was due to the high levels of rainfall (424 mm), which was 190% of the 30-year average for that period.

Wastewater removal efficiency

As there was no rainfall input to the covered wetlands, these figures can be regarded as 100% wastewater evapotranspiration. In terms of wastewater removal alone, these figures compare favourably with the ZDWS evapotranspiration rates recorded by Gregersen and Brix (2001). While evapotranspiration rates of 1087 mm and 1740 mm were recorded in that study, the wastewater loading was only 450 – 600 mm per year, with the remainder consisting of precipitation. A second Danish ZDWS study recorded annual evapotranspiration of 1310 mm, comprised of 520 mm wastewater and 790 mm rainfall (Ministry of Environment and Energy, 2001). The [+ willow + cover] system in this study can thus remove 200-300 mm more wastewater. In the zero-discharge willow lysimeter study of Curneen and Gill (2014) in Ireland, 192 mm of primary or secondary wastewater was applied during the first year, which decreased to 144 mm by the third year of the trial. Rainfall during these first and third years was 788 mm and 839 mm, respectively i.e. 4-5 times greater than the wastewater loading. Curneen and Gill (2015) estimated that removal of 60% of the rainfall loading would allow for the systems to become zero discharge. In this study, the placement of the polytunnel covers led to removal of 100% of the

rainfall loading, i.e. removal of rainfall loading of 944 mm in 2012 and 1,006 mm in 2013. Thus, it can be seen that while the placement of the cover leads to an overall decrease in evapotranspiration as expected, the actual amount of wastewater that can be removed on a per area basis is increased.

Crop factors

Annual crop factor values for the [+ willow + cover] wetland averaged 2.0 in 2012 and 2.1 in 2013. These were lower than the annual crop factor of 2.5 set down by the Danish guidelines or the annual crop factor of 2.2 calculated by Curneen and Gill (2015). However, at 80-90% relative humidity, along with very low wind speed of 0.1 – 0.3 m s^{-1} as would be the case in a polytunnel (Fernández *et al*., 2010), crop factor values are corrected downwards by approximately 0.2 (Allen *et al*., 1998). Applying this correction to the Curneen and Gill (2015) annual crop factor, brings the results recorded in this study closer in line with those recorded by those authors.

Peak crop factor values for the [+ willow + cover] wetlands occurred in October and November of both years. This was slightly later than the peak crop factor values during August – October recorded by Curneen and Gill (2015). This delay was likely due to a prolongation of the growing season, facilitated by elevated temperatures within the polytunnel, combined with a delay in leaf fall due to the low wind speeds within the polytunnels. In addition, crop factor values during the dormant period were frequently over 2.0. These values are higher than the dormant season crop factor values used by Curneen and Gill (2014, 2015), which indicates that the presence of the polytunnel may be particularly important at raising crop factor

levels during the winter months. This would likely be due to the significantly higher maximum temperature inside the tunnel as, for ZDWS in Ireland, temperature has a large influence on evapotranspiration (Curneen and Gill, 2014, 2015).

Willow growth and survival

No clear trends emerged in either the average tree height or tree diameter from 2011 to 2013. In the covered ZDWS, the maximum height that could be attained by the trees was approximately 2.2 m. This height was reached by the tallest trees in each wetland. Willows are known to respond vigorously to coppicing (Caslin *et al*., 2010) and as expected, the overall shoot number per plant increased through each successive coppice cycle.

Willow yield in the current study of 8 – 11.5 odt ha^{-1} was similar to willow yield of 10 - 20 odt ha^{-1} found in willow SRC elsewhere in Ireland (McCracken *et al*., 2001; Larsson *et al*., 2003), and willow yield of 10-12 odt ha^{-1} in ZDWS in Denmark (Gregersen and Brix, 2001). However, declining yield due to annual harvesting was evident once the annual coppice cycle had begun in 2011. Due to the well-established relationship between increasing biomass and increasing evapotranspiration (Białowiec *et al*., 2007a; Guidi, 2008; Curneen and Gill, 2014), such declining yield is of concern to the long term ability of the system to remove wastewater. The presence of willow crop in the [+ willow + cover] wetland led to an additional evapotranspiration of 157 mm in 2012 and 125 mm in 2013 compared to the total evaporation from the [- willow + cover] wetlands. This decrease in relative willow crop contribution (transpiration) from 2012 to 2013 can be explained by the decreased willow crop from 2012 to 2013. Annual harvesting of willows may

compromise the viability of the willow rotation as one season may be too short to adequately replenish the reserves of carbohydrate in the willow stumps after harvest (Ceulemans *et al*., 1996). In contrast, Kopp *et al.* (2001) found that well-adapted willow clones can maintain productivity through ten successive annual harvests. For polytunnel-covered ZDWS, selection of such appropriate clones would be needed for optimum long-term system performance.

Willow survival was 80.7% after three harvests which is similar to the survival rates reported by Kopp *et al.* (1997). The long-term value in using willows in these covered systems is questionable, given that both willow yield and willow survival decreased during the years studied, and due to the ability of the system, i.e. [-willow + cover] wetlands, to accept wastewater in the absence of the trees. Declining yield, along with the need for the annual coppicing of the trees that do survive, and the potential for damage of the polythene cover by the presence of the trees, leads to financial costs and maintenance time that would not feature in tree-less covered system.

Nutrient loading

Initially, concerns had been raised that the use of tertiary wastewater, rather than the advised primary wastewater, may lead to a nutrient loading to the wetlands that would be below the growth requirement of the trees. However, the placement of the cover meant that 100% of the liquid applied was wastewater, as opposed to other studies that would have a very high proportion of rainwater entering. Hence the amounts of nitrogen (104.5 – 259.3 kg N ha^{-1}) and phosphorus (14.5 – 24.4 kg P ha^{-1}) applied were either sufficient or above the levels advised by Caslin *et al.*

(2010) and Wickham *et al.* (2010). In comparison to other ZDWS, the nutrient loadings applied were in excess of those applied to ZDWS by Gregersen and Brix (2001) and zero discharge willow lysimeters by Curneen and Gill (2014).

While other ZDWS used in the domestic setting have received primary wastewater from the septic tank, the use of primary wastewater in a covered i.e. "contained" system, would have public health concerns, even in the event of subsurface spreading of effluent. As a result of these concerns, tertiary effluent is the effluent most likely to gain approval for use in these systems. In this study, a reed bed at Cloyne was used to achieve tertiary status. However, this would not make sense on a domestic scale. Inclusion of a reed bed, where all of the treated wastewater would be directed on to the ZDWS, would somewhat negate the benefit of a cover. In Ireland, the reed bed would gather 1,000 – 2,000 mm of additional water during the course of the year. This would be equal to at least 5 – 10 m^3 of rainfall according the minimum size (5 m^2) allowable for tertiary gravel-based HFSSF or VFSSF by the Code of Practice. Use of a reed bed was acceptable for the purposes of this study, as Cloyne WWTP is not itself zero-discharge and the ZDWS were only supplied with a very small proportion of the overall wastewater flow through the WWTP. Instead, the use of a packaged tertiary system which is not open to rainfall, such as a peat filter, would be advised.

System sizing

To put the performance of the systems in perspective, Table 3.6 shows a comparison between the area demand per person (assuming 100 L wastewater production $person^{-1}$ day^{-1}) required for the willow system according to the Danish

guidelines (120 - 300 m^2 per 100 m^3 wastewater applied), Curneen and Gill (2015) and the results of this study.

Table 3.6 Comparison of wetland area demand per person

	Cloyne ZDWS[a]				Curneen and Gill, 2015[a]	Ministry of Environment and Energy, 2003[b]
	[+ willow + cover]		[- willow + cover]			
	2012	2013	2012	2013		
Area demand person^{-1} (m^2)	55	44	72	52	125	45 – 106

[a] at a loading of 100 L person-1 day-1. [b] Figures used are based on the range contained in the guidelines: 124 – 291 m2 per 100 m^3 wastewater at assumed 100 L person^{-1} day^{-1}.

Both the [+ willow + cover] wetland and the [– willow + cover] wetland have a lower area demand than the results of the Curneen and Gill (2015) paper. It must also be noted that, in the Curneen and Gill (2015) study, the number of overflow days is predicted to be 55 as the authors deemed that ZDWS in Ireland cannot be made zero-discharge due to the high levels of rainfall and the low soil porosity of the backfilled soil. In comparison to the Danish guidelines, the results of the current study are similar to the lower end of the range quoted. This lower range would be found in the south-east of Denmark where rainfall is lowest. However, while the area demand is lower, the feasibility of covering 44 – 72 m^2 per person with polytunnels should be considered. This area is approximately two to three times larger than the polytunnels used here, with associated financial, aesthetic and siting issues. In the case of [- willow + cover] wetlands, financial costs and maintenance costs would be lower as no willow cuttings would need to be installed and no annual coppicing or pest/pathogen control measures would be required.

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Chapter 4 Investigations into the use of a winter-active species, *Petasites fragrans* (Vill.) C. Presi, to increase evapotranspiration in a zero-discharge willow wastewater treatment system

4.1 Abstract

The use of *P. fragrans* (Vill.) C. Presi to achieve increased water loss from a zero-discharge wastewater treatment system was explored. *P. fragrans* is a winter-active, shade-tolerant species, and is often found near river banks and areas susceptible to water inundation. These characteristics indicated its potential suitability as a low-lying intercrop species to increase winter evapotranspiration from a ZDWS. Site characterisations were carried out across a range of different sites where *P. fragrans* was present. *P. fragrans* was found to occur in well-drained sites with very low clay content. Percentage cover and plant dry weight were negatively correlated with light availability. Over a twelve-month study period, percentage cover was found to be highest during winter and spring and to decrease during the summer months, irrespective of shading. In pot trials, evapotranspiration was greatest during the summer months and decreased during the winter months to levels similar to reference evapotranspiration. Annual evapotranspiration increased with increasing wastewater nutrient content. Short-duration waterlogging (2-4 days) and continuous waterlogging were found to affect *P. fragrans* growth, survival and photosynthesis negatively. After six weeks of constant waterlogging, *P. fragrans* survival was zero. The combination of *S. viminalis* and *P. fragrans* in an intercropping trial led to significantly decreased aboveground and belowground biomass production in both species. From the results of these trials it was concluded that the use of *P. fragrans* in a ZDWS setting is not appropriate.

4.2 Introduction

Winter evapotranspiration from full-scale ZDWS in Ireland has been shown to be quite similar to reference evapotranspiration (Curneen and Gill, 2015). During the winter months, the function of the ZDWS is to store all of the incoming wastewater and rainwater.

To increase evapotranspiration during the winter months, the use of a winter-active species *Petasites fragrans* (Vill.) C. Presi was investigated. *P. fragrans* (Winter heliotrope), is a winter-active member of the Asteraceae exhibiting year-round growth in Ireland (O'Mahony, 2009). In Ireland, it is frequently found in damp, shaded soils, and also along river banks suggesting tolerance of occasional inundation. These distribution characteristics indicate the potential suitability of the species for use as a low-lying intercrop species in a ZDWS.

Originating in Libya, Tunisia and Algeria (Toman, 1972), *P. fragrans* was introduced to Ireland in the nineteenth century (O'Mahony, 2009) and spreads rhizomatously to form a dense coverage of approximately 30 cm in height, typically in disturbed habitats such as roadsides (National Roads Authority, 2010). No studies have been published on the evapotranspiration rate of *P. fragrans* heretofore. Monitoring of the evapotranspiration rate over a twelve-month period was therefore considered important to determine the contribution that the species could make to the overall evapotranspiration from a willow-winter heliotrope ZDWS.

Due to the cycle of high summer and low winter evapotranspiration, the wastewater level in a ZDWS follows a cycle of reaching its minimum level at the end of the summer and its maximum level at the end of spring (Curneen and Gill, 2015).

While in Denmark, ZDWS can be designed so that the wastewater level does not breach the soil surface, in Ireland, true "zero discharge" conditions are unlikely to occur due to differences in the distribution of annual rainfall. In Ireland, rainfall ranges from 800 mm in the east to 2,000 mm in the west (Walsh, 2012). In comparison, rainfall in Denmark ranges from 550 mm in the southeast to 900 mm in the west (Frich *et al*., 1997). Curneen and Gill (2015) calculated the number of overflow days in the Irish setting to be approximately 55 days per year. This indicates that frequent, and possibly prolonged, waterlogging of the soil surface of ZDWS would occur. Hence, in the context of using a winter-active species, this species would need to be tolerant of waterlogged conditions. In Ireland, *P. fragrans* is found in a wide variety of habitats, including river banks, which may indicate that the species can tolerate occasional waterlogging.

Previous inter-cropping research involving willow has examined the co-cultivation of willow with mixed tree species including *Juglans nigra* L., *Quercus rubra* L., *Fraximus Americana* L. and *Robinia pseudoacacia* L. which led to increased willow yield (Clinch *et al.*, 2009). In contrast, Wieshammer *et al.* (2007) found that willow leaf biomass production was reduced when willow was intercropped with a low-growing member of the Brassicaceae, *Arabidopsis halleri,* to increase the phytoextraction of Cd and Zn. The use of a winter-active species is a novel attempt to increase winter evapotranspiration from the system. However, it must also be determined whether growing willow in the presence of *P. fragrans* would have any detrimental effects on willow growth, and hence evapotranspiration.

4.2.1 Objectives

The objectives of this study were as follows:

(a) To characterise sites where *P. fragrans* is found.

(b) To investigate the evapotranspiration of *P. fragrans* receiving primary wastewater, tertiary wastewater and rainwater over a twelve-month period.

(c) To investigate the effect of waterlogging on *P. fragrans* growth and survival.

(d) To investigate the intercropping of *S. viminalis* and *P. fragrans.*

4.3 Materials and Methods

4.3.1 Site selection and characterisation

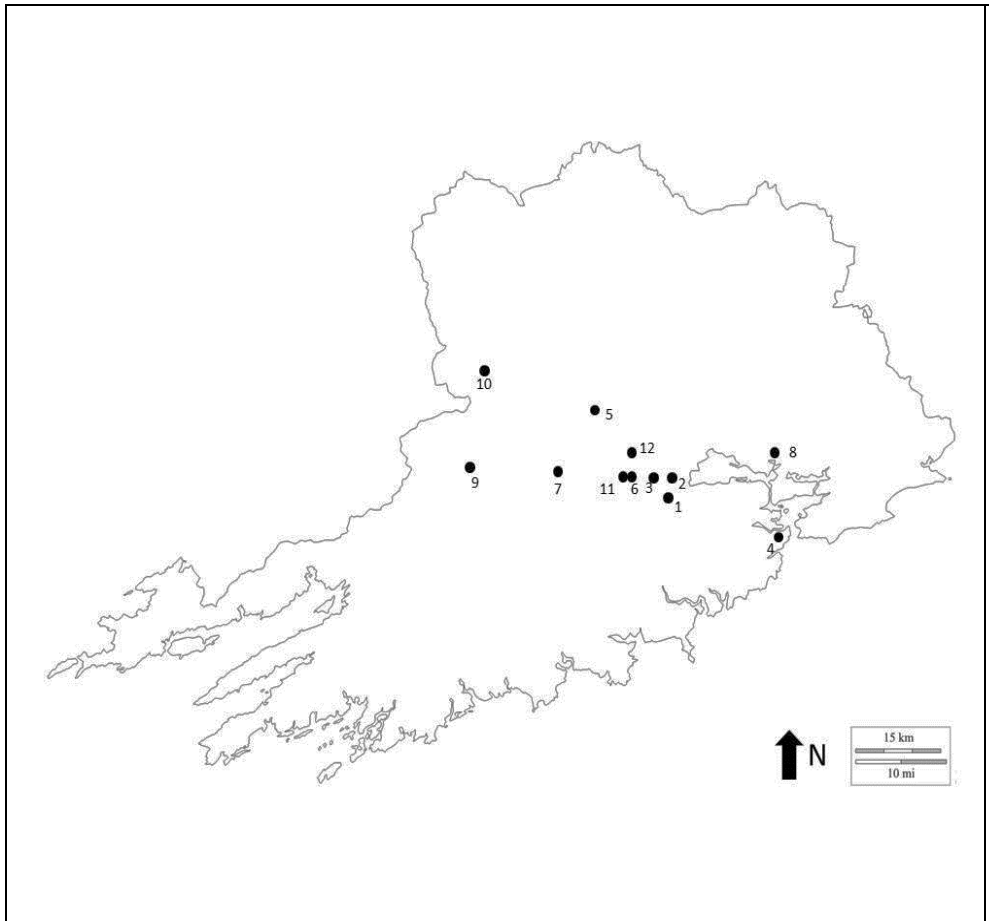
Twelve sites containing *P. fragrans* around Cork city and county were selected (Figure 4.1). Sites were selected to represent a gradient of soil and environmental variables, including altitude, slope, aspect, light availability and moisture content. During January 2015, site characterisations were carried out.

Within each site three 1 x 1 m sampling plots were randomly placed at the start of the trial and permanently marked with wooden poles. The percentage cover of *P. fragrans* within each sampling plot was measured using four 0.25 m^2 meshed quadrats (mesh size = 10 cm x 10 cm) through visual assessment. This was then summed to give the percentage cover for each sampling plot in each site. Shoot density (flowering or vegetative) in each quadrat was also recorded. For plant measurements, a 30 x 30 x 30 cm soil sample was taken from the area immediately adjacent to each 1 x 1 m sampling plot. Rhizome length of all plants in the soil

sample was measured. Five plants were removed and oven dried at 60°C until constant weight was achieved to determine plant dry weight. Soil depth was recorded using a soil auger and measuring tape.

Light availability at the height of *P. fragrans* (approx. 30 cm above ground level) in each 1 x 1 m plot was measured using a SpectroSense2 light meter (Skye Instruments, Llandrindod Wells, UK). A corresponding measurement was made at an adjacent open area. Percentage light availability was then calculated as the ratio of light availability in each quadrat (x 100) divided by the light availability at the open area.

The pH, electrical conductivity and soil texture analysis were determined following the methods of section 3.3.1. The soil moisture content within each plot was measured by taking a 5 cm diameter soil core. Samples were placed in a cooler box and returned to the lab for fresh weight measurement followed by oven-drying the soil until constant weight was achieved (Rowell, 1994). Organic matter was measured using the loss-on-ignition method (Rowell, 1994). Aspect was recorded using a direction-finding compass. Site slope was measured using a clinometer. Altitude was recorded using an online geodatabase (www.maps.ie).



Site number	Location	Grid reference
1	Airport Hill	567107, 566086
2	Atlantic Pond	570537, 572007
3	Cooperage	566530, 572024
4	Crosshaven	578211, 561395
5	Donoughmore	548480, 579842
6	Lee Road	564510, 571512
7	Farnanes	544861, 568003
8	Glounthaune	576346, 573045
9	Macroom	538472, 568559
10	Millstreet	528863, 592105
11	Carrigrohane	563711, 571552
12	Tower	557515, 574619

Figure 4.1 Location of *P. fragrans* study sites in Co. Cork

After January 2015, sites were visited once per month and the percentage cover, soil moisture content and percentage light availability at each site were recorded over a twelve-month period. To compare light levels at the *P. fragrans* sites with the light availability at the ZDWS in Cloyne, percentage light availability was also measured from March to December 2015 in the [+ willow + cover] ZDWS in Cloyne.

4.3.2 Evapotranspiration trial

An evapotranspiration trial was carried out over a twelve-month period starting in March 2015. There were four treatments in this trial: *P. fragrans* irrigated with synthetic primary wastewater, *P. fragrans* with synthetic tertiary wastewater, *P. fragrans* with rainwater, and an unplanted control which was irrigated with rainwater. Rhizomes of *P. fragrans* were gathered from the grounds of the School of BEES and planted in a peat-based potting medium. At the end of a two-week growing period, rhizomes of a similar size were selected and placed in 15 L lysimeters with a gravel layer at the base of each to prevent soil loss through the tap. Three rhizomes (approximately equivalent to the average shoot density found during the site characterisation trial) were planted in each lysimeter. The soil used was the same soil used in section 3.3.1.

Wastewater irrigation was carried out weekly using synthetic domestic primary wastewater (adapted from Rodgers *et al.* (2011))(Table 4.1). The required weight of each constituent was weighed out and mixed using a food blender. The concentrate was then diluted using tap water to the fixed volume of 190 L. Synthetic tertiary wastewater was created by amending the synthetic primary wastewater constituent amounts.

Water quality testing was carried out on the applied synthetic wastewater by the Aquatic Services Unit in the Environmental Research Institute, UCC. The nutrient and organic characteristics of the synthetic primary wastewater and synthetic tertiary wastewater are also shown in

Table 4.2. Lysimeters were irrigated as needed, ensuring that the rainwater/wastewater level was not above the upper surface of the soil.

Table 4.1 Composition of synthetic primary wastewater (adapted from Rodgers *et al.* (2011)) and synthetic tertiary wastewater

Component	Synthetic primary wastewater Amount* (g)	Synthetic tertiary wastewater Amount* (g)
Glucose	85.5	0.95
Yeast extract	7.13	0.08
Dried milk	28.5	0.03
Urea	7.13	2.38
NH_4Cl	14.25	4.75
$Na_2HPO_4.12H_20$	23.75	7.92
$KHCO_3$	11.88	3.96
$NaHCO_3$	30.88	10.29
$MgSO_4.7H_2O$	11.88	3.96
$FeSO_4.H_2O$	0.78	0.26
$CaCl_2.6H_2O$	0.05	0.02
Bentonite	10.64	3.58

***dissolved in 190 L water**

Table 4.2 Water quality parameters of the synthetic primary wastewater

Component	Synthetic primary wastewater concentration	Synthetic tertiary wastewater concentration
BOD_5	475.0 (mg O_2 L^{-1})	9.5 (mg O_2 L^{-1})
COD	621.0 (mg O_2 L^{-1})	40.0 (mg O_2 L^{-1})
Total Nitrogen	53.1 (mg N L^{-1})	7.0 (mg N L^{-1})
Orthophosphate	10.2 (mg P L^{-1})	3.4 (mg P L^{-1})

Evapotranspiration was calculated using the water balance method, by measuring the drainage from the lysimeters once every one to two weeks:

$$ET = Q - D \qquad \textbf{Equation 4.1}$$

where ET = evapotranspiration, Q = wastewater irrigation and D = wastewater drainage. Lysimeters were located outdoors but under the shelter of mini-polytunnels at the School of BEES, UCC (Figure 4.2). This was done to remove the effects of incident rainfall due to the small volume of the lysimeters. To facilitate air circulation, the bases of the polytunnels were raised off the ground on blocks and a 20 cm strip of polythene was removed just above the base of each polytunnel. There were twelve lysimeters under each polytunnel, with three lysimeters of each treatment per polytunnel. The overall replicate number for each treatment was four, with each replicate consisting of three lysimeters underneath the same polytunnel. Reference evapotranspiration data were taken from the Met Éireann site at Cork Airport (6 km away). Monthly crop factor values were calculated for each of the twelve months.



Figure 4.2 Lysimeters containing *P. fragrans* (left) and mini-polytunnels (right) used during the transpiration trial.

4.3.3 Waterlogging trial

To examine the effect of saturated soil conditions on the growth and survival of *P. fragrans* rhizomes a waterlogging trial was carried out at the School of BEES, UCC. There were two parts to this trial. The first part consisted of four treatments: constant submergence for seven days a week, submergence for four continuous days out of seven, submergence for two continuous days out of seven and a control which was irrigated with synthetic primary wastewater every 1-2 days. Synthetic primary wastewater (section 4.3.2) was used for submergence. Rhizomes were prepared as above (section 4.3.2) and placed in 17.5 cm pots with one rhizome per pot. Submerged conditions were brought about by placing these pots into larger 7.5 L pots which contained waterproof plastic bags filled with primary wastewater. When not submerged, pots were placed adjacent to the submergence pot. The same soil was used as in section 4.3.2. Plants were located outdoors, not under the cover of a polytunnel in a replicated randomised block design. There were fifteen replicates per treatment, each replicate represented by one pot. The trial was run for six weeks. At the end of the trial the aboveground dry weight, belowground dry weight and survival of each plant were recorded.

In the second part of the trial, chlorophyll fluorescence was measured in the fifteen control plants from part one ("control") and on an additional fifteen rhizomes which underwent a 10-day waterlogging treatment ("waterlogging"). Non-destructive measurement of chlorophyll fluorescence was made on the uppermost fully developed leaf on each *P. fragrans* plant using an OS-30p chlorophyll fluorometer and leaf clips (Opti-Sciences Inc. NH, USA).

Maximum efficiency of photosystem II (PSII) was calculated as:

$$(F_m - F_o)/F_m = F_v/F_m \quad \textbf{Equation 4.2}$$

Where F_o = minimum fluorescence in the dark-adapted state, F_m = maximum fluorescence in the dark-adapted state and F_v = variable fluorescence ($F_m - F_o$). Beginning on the second week of the first part of the trial, F_v/F_m was recorded during ten days of waterlogging and for ten days following the cessation of waterlogging. The same measurements were carried out on the control treatment during the twenty-day period. Leaves were dark adapted for twenty minutes prior to measurement. Measurements were made between 9am and 11am every second day during the course of the trial.

4.3.4 Intercropping trial

To examine the effect of intercropping *S. viminalis* with *P. fragrans*, an intercropping pot trial was conducted at the School of BEES, UCC. There were three treatments: *S. viminalis* only ("willow monocrop"), *P. fragrans* only ("*P. fragrans* monocrop") and an *S. viminalis* and *P. fragrans* combination ("intercrop"). In both of the *P. fragrans* treatments, thirteen *P. fragrans* rhizomes were planted in each 30 L pot (surface area = 0.13 m^2) filled with a multi-purpose potting compost, as this shoot density reflected the average shoot density found during the field site characterisations. Rhizomes were prepared as described in section 4.3.2. At the start of the trial, each of the pots containing *P. fragrans* was standardised for total *P. fragrans* fresh weight, ensuring that there was an equal total fresh weight of *P. fragrans* in each pot. For the *S. viminalis* treatments, rods of *S. viminalis* (8 mm

diameter) taken from the field site in Cloyne were cut to a length of 25 cm and soaked overnight. Ten days after *P. fragrans* planting, two *S. viminalis* cuttings were planted in each of the willow monocrop pots and the intercrop pots. This was thinned to one willow cutting per pot just after willow shoot emergence. Pots were arranged in a replicated randomised block design, with a replicate number of nine, one pot per replicate. All pots were irrigated weekly with 2 L of synthetic primary wastewater (section 4.3.2).

Height and basal diameter of the leading willow shoot were measured weekly during the trial which lasted for twenty weeks (May to September 2015). At the end of the trial, willow root and shoot dry weights were recorded after drying for three days at 60 °C. For each pot containing *P. fragrans*, the overall aboveground dry weight, belowground dry weight and percentage cover at the end of the trial were recorded.

Intercropping advantage (IA) on the aboveground data was assessed according to:

$$IA = \frac{\text{Aboveground intercrop weight}}{[(\text{monocrop willow aboveground weight}/2) + (\text{monocrop } \textit{P. fragrans} \text{ aboveground weight}/2)]}$$

Equation 4.3

4.3.5 Statistical analysis

All data were checked for normality and homogeneity of variance prior to analysis and transformed to achieve normality if necessary. Pearson's correlation coefficient was used to investigate relationships between environmental and soil variables and plant responses (collected during January 2015). Percentage cover and rhizome density data were square root transformed to normalise and plant dry weight data were log transformed to normalise prior to correlation analysis. Canonical

Correspondence Analysis (CCA) (ter Braak, 1987) was used to examine relationships between *P. fragrans* characteristics and the measured environmental and soil variables (taken during January 2015). CCA was chosen over other multivariate approaches such as Principal Component Analysis and Redundancy Analysis due to unimodal responses of *P. fragrans* growth characteristics to environmental and soil variables. Environmental and soil data were standardised to a mean of zero prior to analysis (ter Braak, 1987). The four plant variables were transformed, where needed. Percentage silt was not included in the CCA analysis due to covariance with percentage sand. CCA analysis was carried out using MVSP (Kovach Computing, Anglesey, Wales). The ordination plot was interpreted following ter Braak (1987) and Kohler *et al.* (2005). CCA eigenvalues were assessed for significance using the Monte Carlo simulation test with 199 random permutations using Canoco 4 (Cajo ter Braak, Wageningen University, Netherlands).

Annual evapotranspiration trial data were normalised by square root transformation and analysed using a one-way ANOVA. A one-way ANOVA was also used to examine the measured growth parameters of the waterlogging trial. Belowground biomass data were log transformed prior to analysis. Multiple comparisons were carried out using the Tukey test. Analysis (unpaired t-test) of the F_v/F_m waterlogging data were carried out at each time point to test for significant differences due to waterlogging. Waterlogging survival data were analysed using two-way χ^2 analysis. For the intercropping trial, mean differences in growth between the monocrop and intercrop pots were compared using an unpaired t-test. Willow shoot and root biomass, height, shoot number and percentage cover data

were square root transformed prior to analysis to approximate to normality. *P. fragrans* belowground dry weight data were also square root transformed prior to analysis to approximate to normality. Descriptive statistics are presented as non-transformed values. Statistical analyses were carried out using SPSS, unless otherwise stated.

4.4 Results

4.4.1 Site characterisation

Plant variables in January 2015

During the site characterisation of January 2015, mean *P. fragrans* percentage cover varied between sites, from a low of 39.2% at Donoughmore to a high of 90.2% at Atlantic Pond (Table 4.3).

Table 4.3 Mean ± SE *P. fragrans* variables measured in January 2015

Site	Percentage cover	Shoot density (plant m^{-2})	Rhizome length (cm)	Total plant dry weight (g)
Airport Hill	60.8 ± 17.45	111.1 ± 23.13	14.6 ± 1.08	4.0 ± 0.47
Atlantic Pond	90.2 ± 0.67	92.6 ± 20.26	13.8 ± 2.14	5.0 ± 0.48
Carrigrohane	82.3 ± 7.44	92.6 ± 24.29	12.2 ± 1.72	5.3 ± 0.74
Cooperage	65.2 ± 6.49	144.2 ± 31.65	26.0 ± 3.09	4.3 ± 1.01
Crosshaven	54.0 ± 14.02	37.0 ± 7.41	21.1 ± 1.08	4.7 ± 1.05
Donoughmore	39.2 ± 13.42	66.7 ± 0.00	15.6 ± 2.89	4.4 ± 1.19
Farnanes	53.3 ± 6.65	85.2 ± 3.70	13.6 ± 1.52	5.9 ± 0.35
Glounthaune	51.8 ± 14.97	181.5 ± 24.29	16.0 ± 2.61	2.3 ± 0.51
Lee Road	58.8 ± 8.59	127.3 ± 30.14	13.8 ± 1.57	3.7 ± 0.96
Macroom	39.7 ± 7.33	74.1 ± 7.41	16.5 ± 1.42	3.2 ± 0.10
Millstreet	59.8 ± 14.66	118.5 ± 22.53	14.1 ± 0.96	3.5 ± 0.51
Tower	46.8 ± 5.70	74.1 ± 19.60	16.0 ± 0.65	3.6 ± 0.05

Shoot density was highest in Glounthaune (181.5 shoots m^{-2})(Table 4.3). Mean rhizome length varied from 12 to 20 cm with only sites at Cooperage and

Crosshaven showing longer rhizome lengths, of 26.0 cm and 21.1 cm, respectively. Mean plant dry weight varied from 2.3 g in Glounthaune to 5.9 g in Farnanes.

Soil and environmental variables in January 2015

Of the twelve sites, five of the sites experienced 100% light availability. For the remaining seven sites, where shading did occur, light availability varied from 4.6% in Crosshaven to 58.6% in Lee Road (Table 4.4). Percentage light availability was significantly negatively correlated to percentage cover ($r = -0.60$; $p < 0.05$) and plant dry mass ($r = -0.60$; $p < 0.05$)(Table 4.5). The most common aspect was south-facing which occurred at five of the twelve sites.

Slope varied considerably, from 0° in Glounthaune to 42° in Millstreet. However, there was no significant relationship between slope and any of the four plant variables (Table 4.5). Altitude varied across the twelve sites from 153 m in Donoughmore to 5 m in Glounthaune. Altitude was not significantly correlated to any of the measured plant variables. The pH values recorded ranged from pH 6.2 in Donoughmore to pH 7.7 in Glounthaune, with the Pearson's correlation coefficient with percentage cover approaching statistical significance ($r = +0.565$; $p = 0.056$). Electrical conductivity varied from 130.2 µS cm^{-1} in Tower to 257.5 µS cm^{-1} in Crosshaven. A significant positive correlation between EC and rhizome length was found ($r = 0.628$; $p < 0.05$).

Table 4.4 Mean ± SE (where appropriate) environmental variables and soil variables of the twelve study sites in January 2015.

Site	Environmental variables				Soil variables							
	Light (%)	Aspect	Slope (degrees)	Altitude (m)	pH	$EC_{1:5}$ ($\mu S\ cm^{-1}$)	Moisture content (%)	Organic matter (%)	Sand (%)	Silt (%)	Clay (%)	Soil depth (cm)
Airport	100 ± 0.00	W	18	143	6.4 ± 0.32	222.1 ± 28.07	27.1 ± 1.25	10.7 ± 0.34	40.5	58.3	1.2	12.7
Atlantic Pond	9.0 ± 0.49	NW	5	8	7.5 ± 0.24	204.5 ± 9.74	22.1 ± 1.95	8.9 ± 1.47	71.8	27.5	0.7	42.0
Carrigrohane	52.3 ± 7.02	NE	22	8	7.6 ± 0.07	164.1 ± 31.19	24.0 ± 2.99	12.5 ± 1.74	73.0	25.3	1.7	33.0
Cooperage	12.7 ± 4.95	W	40	13	7.4 ± 0.30	243.3 ± 53.86	25.6 ± 2.10	20.2 ± 2.75	69.6	29.6	0.6	60.0
Crosshaven	4.6 ± 0.75	N	20	12	7.6 ± 0.08	257.5 ± 7.76	25.5 ± 0.66	31.7 ± 17.36	71.4	27.8	0.9	33.7
Donoughmore	100 ± 0.00	S	38	153	6.2 ± 0.16	130.7 ± 16.17	19.7 ± 1.51	6.8 ± 1.14	89.9	10.8	0.2	26.3
Farnanes	28.9 ± 10.60	S	38	96	6.9 ± 0.15	158.6 ± 20.86	25.0 ± 0.61	10.9 ± 1.56	64.2	35.0	0.8	35.0
Glounthaune	100 ± 0.00	S	0	5	7.7 ± 0.03	197.7 ± 21.53	35.5 ± 2.76	10.5 ± 0.26	81.6	17.7	0.7	15.7
Lee Road	58.6 ± 1.07	S	26	6	7.0 ± 0.31	135.7 ± 20.34	13.4 ± 5.22	7.6 ± 1.34	80.7	18.9	0.4	35.0
Macroom	100 ± 0.00	NE	30	58	6.7 ± 0.08	163.8 ± 22.82	25.0 ± 2.67	9.4 ± 1.51	83.7	15.7	0.6	50.7
Millstreet	39.3 ± 1.32	W	42	112	7.5 ± 0.02	155.7 ± 4.48	20.5 ± 2.38	7.4 ± 0.91	76.1	23.3	0.5	35.3
Tower	100 ± 0.00	S	25	17	7.0 ± 0.12	130.2 ± 24.12	21.2 ± 0.51	6.5 ± 0.40	53.7	44.5	1.7	28.7

Similarly, a significant positive correlation between organic matter and rhizome length was found ($r = 0.683$; $p < 0.05$). No significant correlations between moisture content and any of the plant variables were found. Most sites had a high sand content (>50%) with only the site on Airport Hill having a lower sand percentage (40.5%). All sites had a very low clay content (< 2% clay in all sites). Soil depth was reasonably shallow on all sites and, in general, varied between 10 cm and 40 cm in depth. Only sites at the Cooperage (60.0 cm) and Macroom (50.7 cm) were deeper. No significant correlations were found between the plant variables and any of the soil texture classes or soil depth.

Table 4.5 Pearson correlation coefficients between *P. fragrans* parameters and measured environmental and soil variables across twelve sites. Correlation coefficients in bold are significant at $p < 0.05$.

	Plant variables			
Environmental and soil variables	**Percentage cover**	**Shoot density**	**Rhizome length**	**Dry weight per plant**
Light availability	**-0.598**	0.142	-0.265	**-0.601**
Slope	-0.353	-0.194	0.185	0.257
Altitude	-0.396	-0.150	-0.226	0.090
pH	0.565	0.258	0.141	-0.036
$EC_{1:5}$	0.341	0.028	**0.628**	0.089
Moisture content	-0.076	-0.164	0.461	-0.081
Organic matter	0.104	-0.346	**0.683**	0.259
Sand	-0.219	0.032	0.015	-0.198
Silt	0.207	-0.035	-0.010	0.200
Clay	0.285	-0.178	-0.201	0.186
Soil depth	0.155	-0.086	0.456	0.230

Canonical Correspondence Analysis is frequently used in ecological studies and has been previously used to describe the growth and distribution of rhizomatous species in response to environmental gradients (He *et al.*, 2012). The plot of the

CCA analysis describes the variation in the four plant variables in relation to the measured soil and environmental parameters at the twelve study sites (Figure 4.3). Eigenvalues for axes one and two were 0.018 and 0.002, respectively; Monte-Carlo simulation procedure $p > 0.05$. Axis 1 was mainly composed of a slope, altitude and pH gradient and explained 85.6% of the variation in *P. fragrans* parameters. Axis 2 was mainly composed of a light and organic matter gradient and explained 8.8% of the variation in *P. fragrans* parameters. The arrow for moisture content is short indicating that moisture content across the gradient present at the twelve sites did not influence *P. fragrans growth.* The ordination results indicate a positive association of shoot density with increasing available light. Rhizome dry weight is pushed to the edge of the ordination space along the organic matter-light gradient. No clear associations were found for either rhizome length or percentage cover.

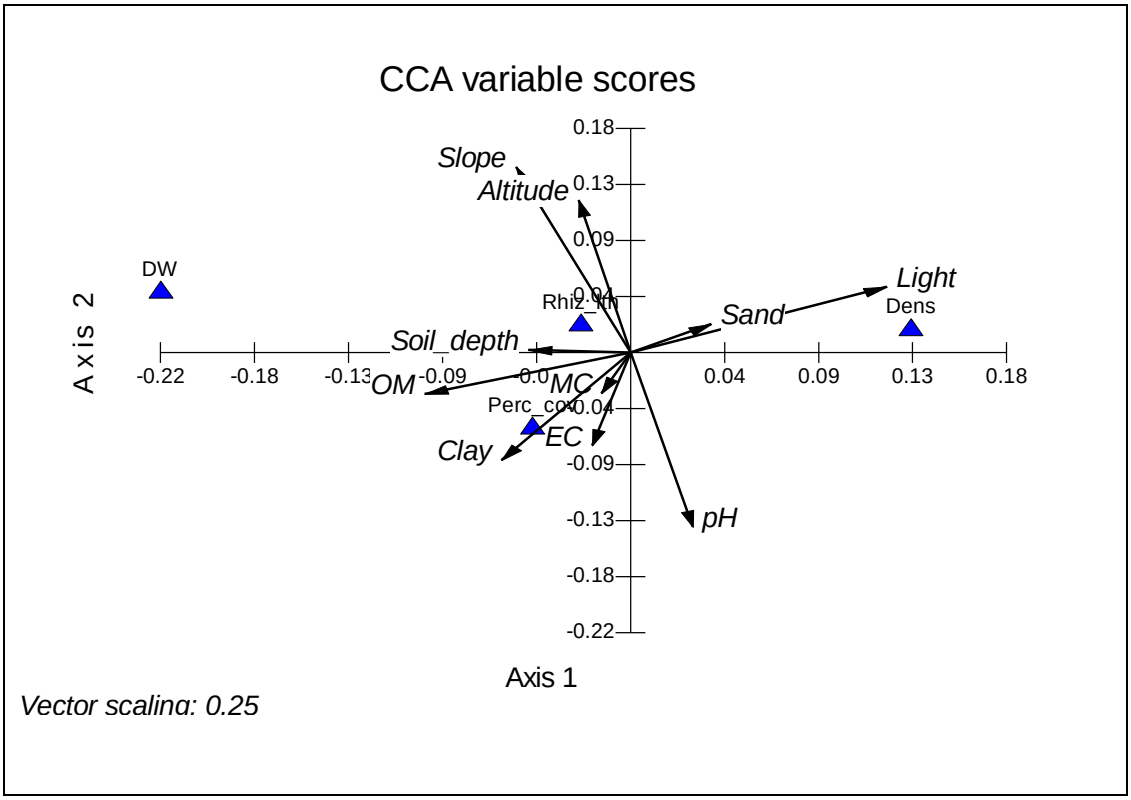


Figure 4.3 CCA ordination diagram. *P. fragrans* variables: dry weight per plant (DW), percentage cover (Perc_cover), shoot density (Dens), rhizome length (Rhiz_lth).

Temporal changes

The highest levels of percentage cover occurred during the period February – April (Figure 4.4 (A)). Percentage cover then declined throughout the summer, reaching its lowest values in July-August. Beginning in September, percentage cover began to increase during the late autumn and winter months. No clear trend in soil moisture across the twelve-month period was observed, with values in general varying from 20 to 30% moisture content across the year (Figure 4.4 (B)).

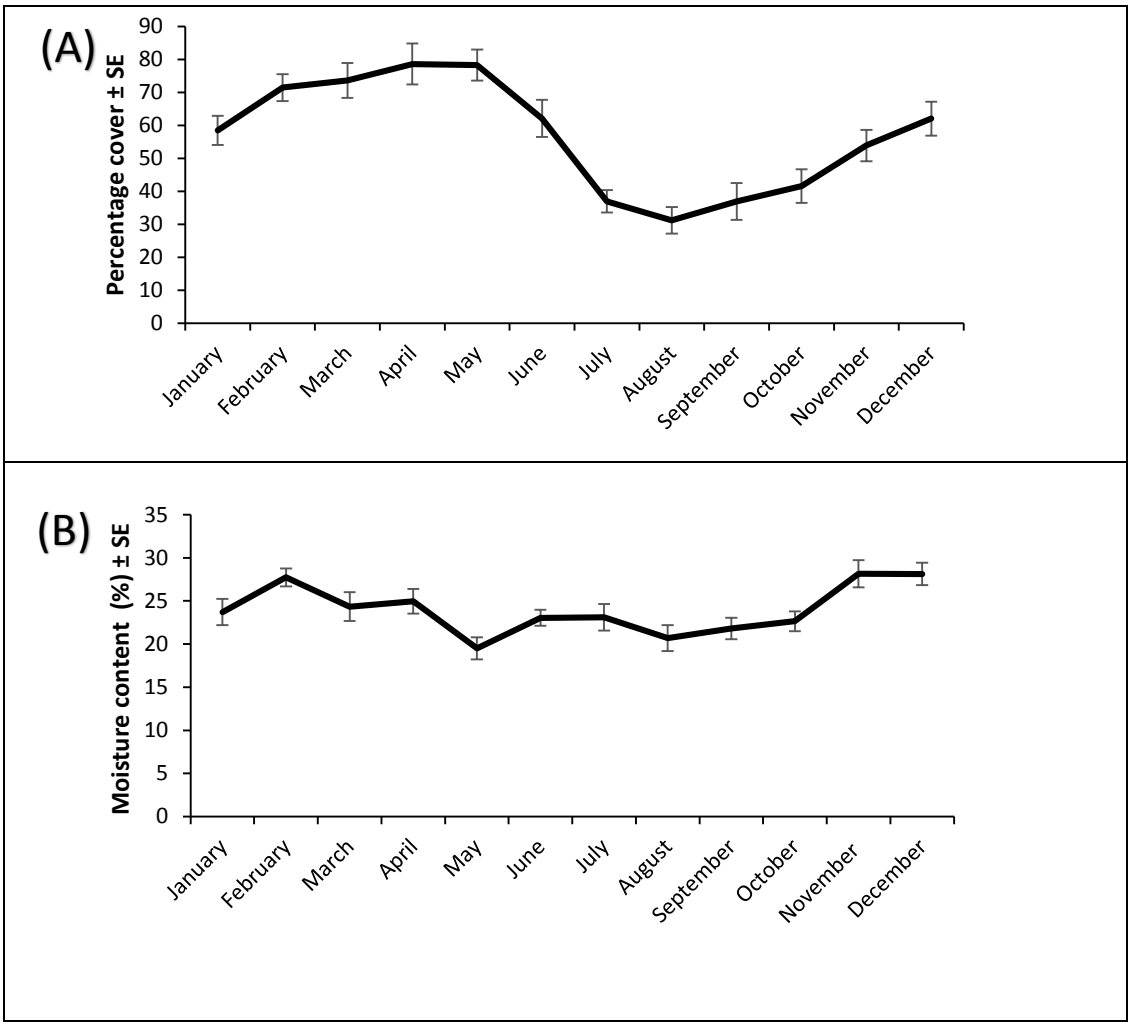


Figure 4.4 Temporal variation in mean ± SE (A) monthly percentage cover of *P. fragrans* and (B) mean ± SE monthly percentage soil moisture content. Points represent mean values of twelve sites.

Monthly light availability varied over the course of the year at the sites where shading occurred (Figure 4.5 (A)). Light availability during the summer months (May – August) was quite low, and, in general, less than 5% of the available daylight. Light availability was higher from January to April and from November to December when leaf fall from accompanying trees had occurred. A similar pattern of light availability occurred in the [+ willow + cover] ZDWS in Cloyne (Figure 4.5 (B)). Light availability during the summer months was quite low. During July and August, less than 2% of the available light was recorded beneath the willow canopy in these wetlands. Approximately 20% of the reduction in light in the [+ willow + cover] wetlands was due to the presence of the polythene cover. The remainder was due to the presence of the willow canopy.

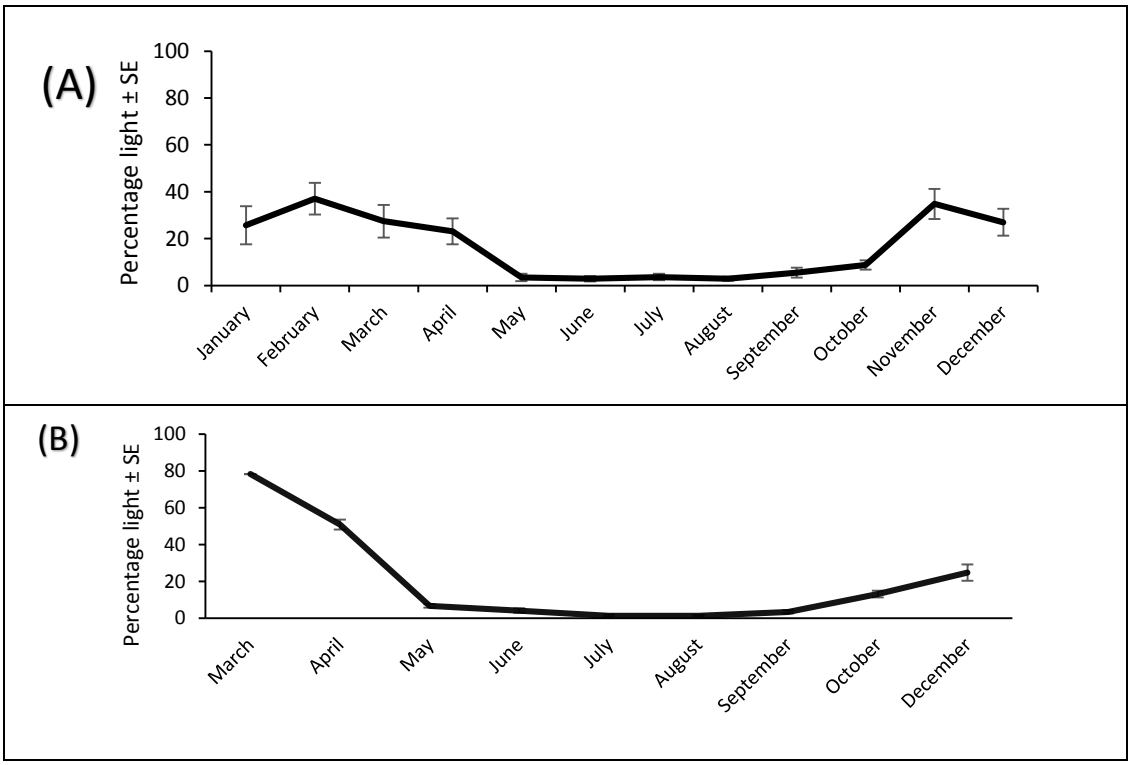


Figure 4.5 Mean ± SE monthly light availability at each of the sites where shading occurred (A) and at the ZDWS site in Cloyne during 2015 (B).

4.4.2 Evapotranspiration trial

For the first three months of the trial, evapotranspiration rates for primary, tertiary and rainwater lysimeters were similar (Figure 4.6). Evaporation rate for the control treatment was slightly lower. Evapotranspiration rates for the rainwater and control treatments closely followed the reference evapotranspiration throughout the twelve-month period. Lysimeters that received primary or tertiary wastewater had higher evapotranspiration levels during the summer months. *P. fragrans* plants receiving primary effluent had the highest daily evapotranspiration which peaked in September at 3.8 mm day^{-1}. Plants receiving tertiary wastewater peaked at 2.9 mm day^{-1} in August. Evapotranspiration rates for the primary and tertiary wastewater treatment began to fall in October, to levels similar to the rainwater and control treatments. Evapotranspiration from November to February was similar to the reference evapotranspiration in all treatments.

There were significant differences in annual evapotranspiration rate between all four treatments over the twelve-month period ($F(3,12) = 138.52$; $p < 0.001$) (Table 4.6). Lysimeters that received primary wastewater showed significantly higher evapotranspiration than all other treatments (659 mm). Lysimeters that received tertiary wastewater exhibited approximately 100 mm less annual evapotranspiration (556 mm) than lysimeters that had received primary wastewater. Lysimeters that received rainwater had a total evapotranspiration of 446 mm, compared to 402 mm for the unplanted control lysimeters. The total reference evapotranspiration over the twelve month period was 482 mm.

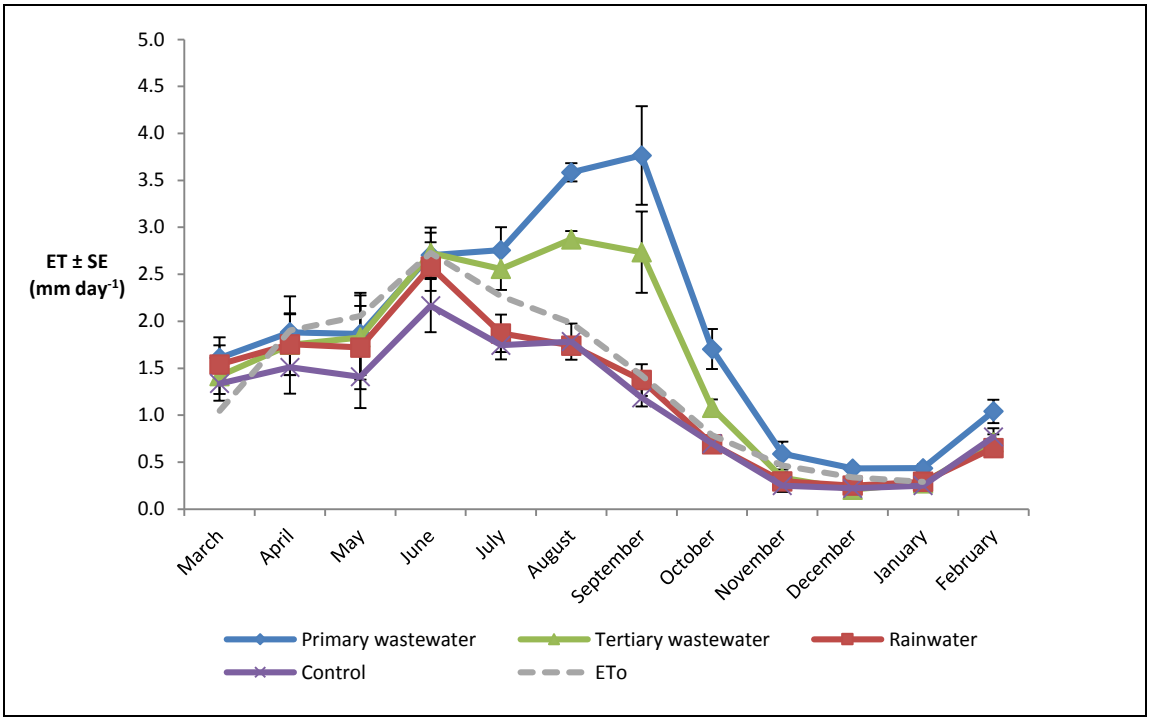


Figure 4.6 Mean ± SE daily evapotranspiration from *P. fragrans*-planted lysimeters and unplanted control lysimeters, and reference evapotranspiration over a twelve-month monitoring period.

Table 4.6 Mean ± SE annual evapotranspiration from *P. fragrans*-planted lysimeters and unplanted control lysimeters over a twelve-month monitoring period. Any two samples that share common letters are not significantly different using the Tukey test ($p > 0.05$).

Treatment	Mean ± SE annual evapotranspiration (mm)
Primary	659 ± 7.6 a
Tertiary	556 ± 16.1 b
Rainwater	446 ± 13.0 c
Control	402 ± 3.0 d

Monthly k_c values were highest during the late summer months and autumn and peaked at 2.6 for plants receiving primary wastewater and 1.9 for plants receiving tertiary wastewater in September (Figure 4.7). Plants receiving rainwater showed k_c values close to 1.0 for the duration of the trial. Annual k_c values ranged from 0.9 for plants that received rainwater, to 1.4 for plants that received primary wastewater (Table 4.7).

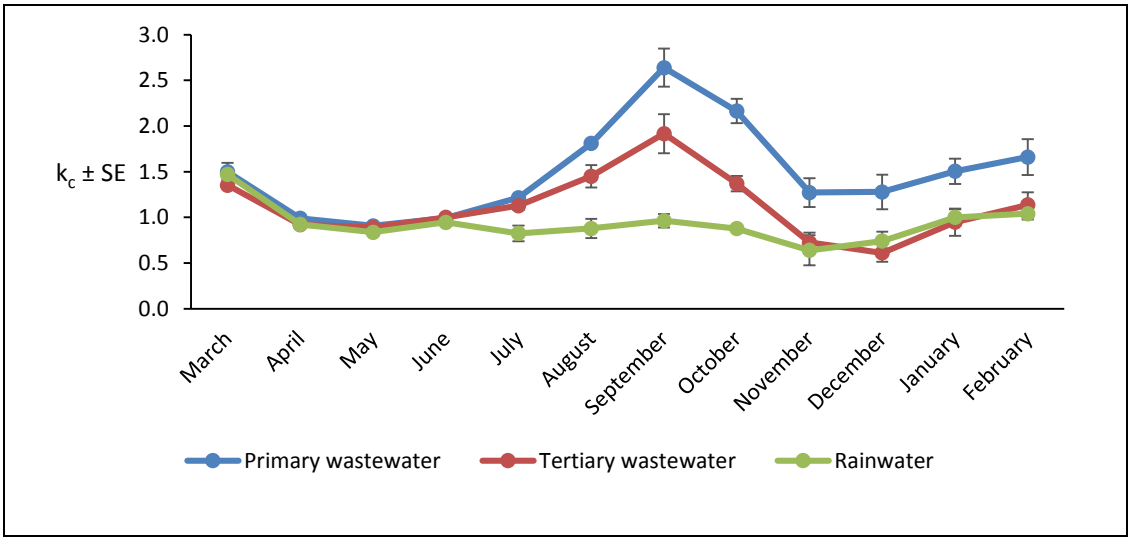


Figure 4.7 Mean ± SE monthly k_c values from *P. fragrans*-planted lysimeters over a twelve-month monitoring period.

Table 4.7 Mean ± SE annual k_c values from *P. fragrans*-planted lysimeters over a twelve-month monitoring period

Treatment	Annual k_c
Primary	1.4 ± 0.01
Tertiary	1.1 ± 0.03
Rainwater	0.9 ± 0.02

4.4.3 Waterlogging trial

Winter heliotrope plants growing in free-draining conditions had the largest final aboveground biomass of 3.76 g plant^{-1} at the end of the trial (Figure 4.8). Aboveground biomass of the two-day, four-day and constant waterlogging treatments were lower, but there were no significant differences between any of the four treatments ($F(3,56) = 2.31$, $p > 0.05$). There were significant differences in the belowground biomass of plants between all four treatments ($F(3,56) = 44.67$, $p < 0.001$). Plants growing in free-draining conditions had the largest belowground biomass of 10.62 g plant^{-1}. The general trend was for the belowground biomass to

decrease with increasing duration of waterlogging. Plants growing in the constantly flooded conditions had the lowest final belowground biomass of 1.48 g plant^{-1}.

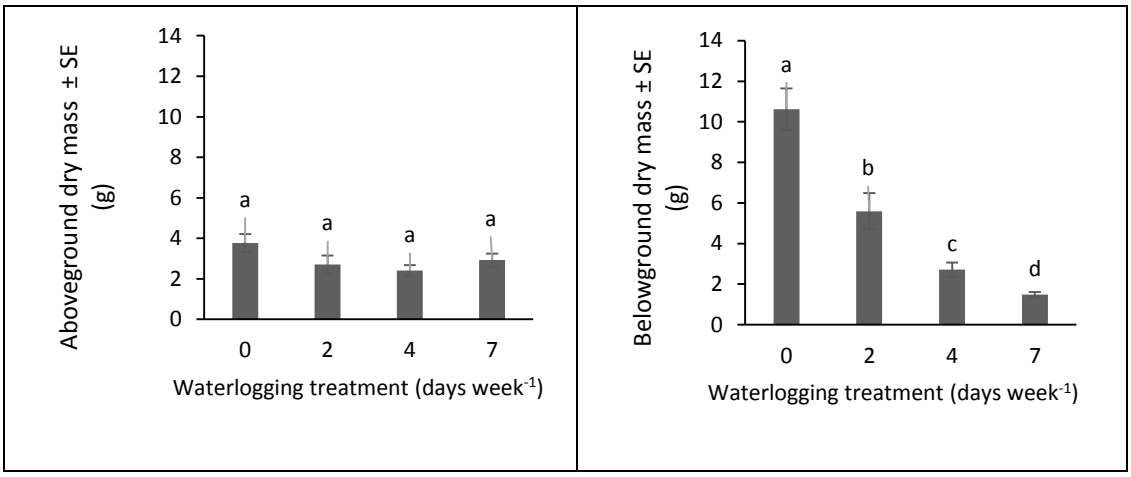


Figure 4.8 Mean ± SE aboveground (left) and belowground (right) final dry mass of *P. fragrans* plants. Any samples within a figure which share a common letter are not significantly different using the Tukey test (p > 0.05).

Plants grown in the 0 days waterlogging showed 100% survival at the end of the trial (Table 4.8). No plants grown in constant submergence survived the six-week trial. A significant association between waterlogging and survival was found (χ^2 = 34.91, 3 df; p < 0.001).

Table 4.8 Survival of fifteen *P. fragrans* plants in each treatment at the end of the waterlogging trial

Treatment (days week^{-1} waterlogged)	**0**	**2**	**4**	**7**
Survival (no. plants)	15	10	4	0

A significant difference in chlorophyll fluorescence between waterlogged and control plants was found after two days of the waterlogging treatment (t = 2.59, 28 df; p < 0.05) (Figure 4.9). By day ten (the last day of the waterlogged conditions), control plants had a mean F_v/F_m ratio of 0.79 and waterlogged plants had a mean

F_v/F_m ratio of 0.53. After removal of waterlogging conditions, the previously waterlogged plants did not recover in terms of F_v/F_m. The average F_v/F_m values continued to decline and reached a mean final F_v/F_m ratio of 0.44 after twenty days.

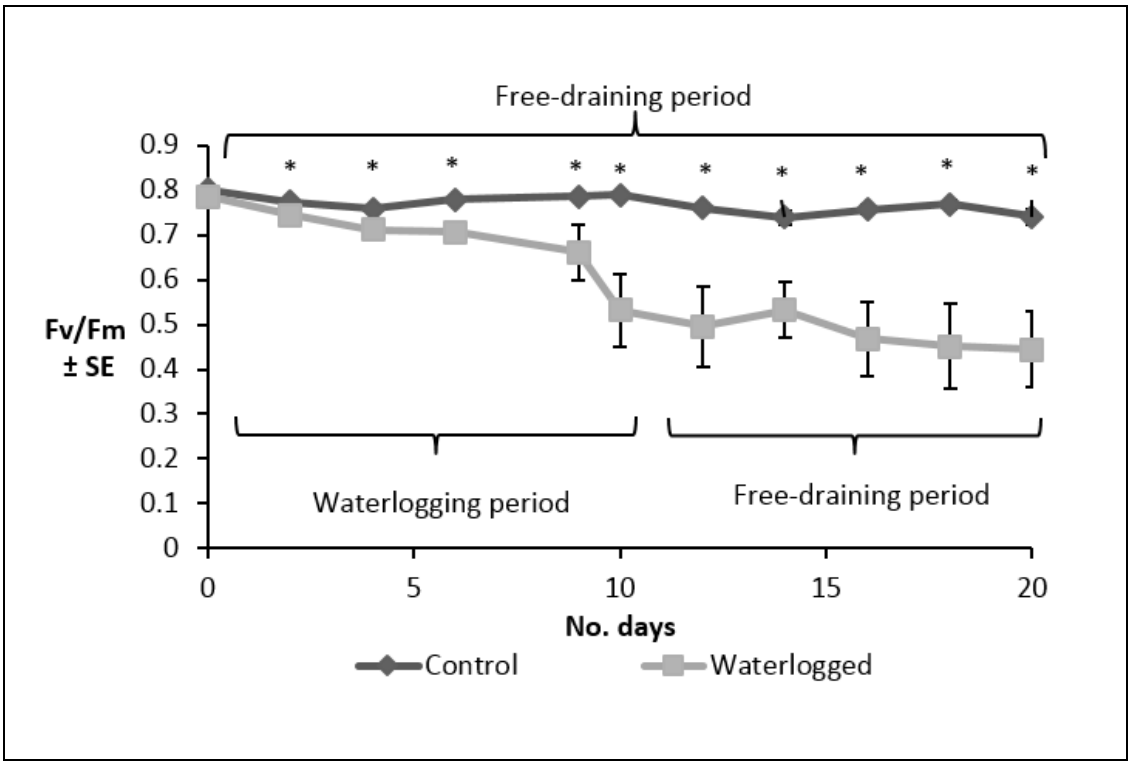


Figure 4.9 Mean ± SE chlorophyll fluorescence measurements of *P. fragrans* during ten days of waterlogged conditions followed by ten days of free-draining conditions.

Control plants were not subjected to waterlogged conditions during the 20 days of the trial. * denotes significant differences ($p < 0.05$) between waterlogging and control plants on the same day.

4.4.4 Intercropping trial

Small differences in willow height and diameter between willow monocrop and willow-winter heliotrope intercrop began to emerge after five weeks (Figure 4.10). However, there were no significant differences in final height or final diameter at the end of the trial.

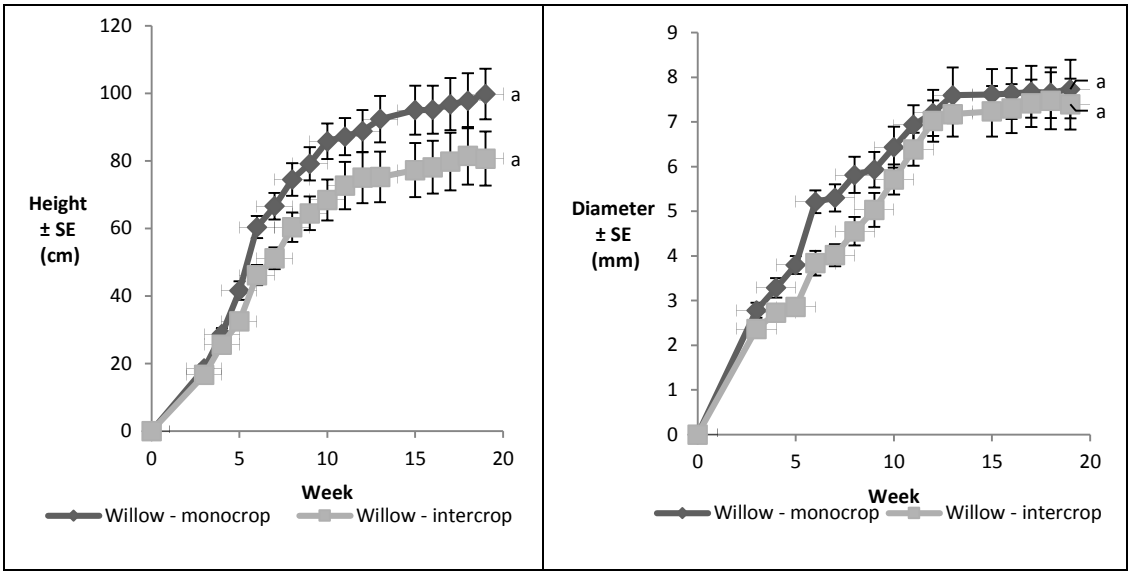


Figure 4.10 Mean ± SE weekly willow height and diameter during the course of the intercropping trial. Any samples which share a common letter are not significantly different using the Tukey test (p > 0.05).

Willow shoot dry mass, root dry mass and final shoot number were significantly greater in the willow monocrop than the intercrop (Table 4.9). Willow shoot biomass in the willow monocrop was more than twice that in the intercrop. The willow monocrop root biomass was approximately three times larger than that of the willow intercrop. Willows showed a significantly higher number of shoots in the willow monocrop than in the intercrop. For *P. fragrans*, the aboveground biomass and belowground biomass were significantly larger in the *P. fragrans* monocrop than in the intercrop (Table 4.9). Aboveground biomass was approximately twice that in the monocrop compared to the intercrop. Belowground *P. fragrans* biomass was 50% greater in the *P. fragrans* monocrop than in the intercrop, though there was no significant difference in *P. fragrans* final percentage cover between the monocrop and intercrop treatments. The intercropping advantage (IA) was calculated as 0.93, indicating that intercropping conferred a disadvantage on aboveground biomass production.

Table 4.9 Mean ± SE final *S. viminalis* and *P. fragrans* measurements in the mono- and intercrop treatments. Any two samples with column which share a common letter are not significantly different using the Tukey test (p > 0.05).

	Willow shoot dry mass (g)	Willow root dry mass (g)	Willow shoot number	*P. f.* aboveground dry mass (g)	*P. f.* belowground dry mass (g)	*P. f.* % cover
Monocrop – willow	30.6 ± 6.4 a	29.9 ± 4.8 a	5.4 ± 0.7 a	-	-	-
Intercrop	13.9 ± 2.3 b	10.9 ± 2.8 b	2.8 ± 0.2 b	3.5 ± 0.6 a	27.3 ± 4.2 a	31.9 ± 5.6 a
Monocrop – *P. fragrans*	-	-	-	6.5 ± 0.6 b	40.8 ± 3.1 b	45.1 ± 4.6 a

4.5 Discussion

Site characterisation

P. fragrans is known to form dense carpets of cover (National Roads Authority, 2010). High percentage cover was found during this trial, but only during winter and spring. The percentage cover varied throughout the twelve-month period, with the lowest cover occurring during July to September. While the site characterisation in January found a significant negative relationship between light availability and percentage cover, during the summer months the percentage cover on the shaded sites followed the same trend as the sites where light availability was 100%. This reduction in percentage cover is not likely to be related to light availability during the summer months. Instead, the trend can be explained by considering the natural range of *P. fragrans.* This species is native to Libya, Tunisia and Algeria (Toman, 1972) and was introduced to Ireland in the nineteenth century (O'Mahony, 2009). This reduction in cover during the summer months is likely due to its original growth cycle in North Africa, whereby a reduction in percentage cover was needed as a drought-escaping strategy during the hot, dry summer months. A higher leaf area is known to lead to higher evapotranspiration (Persson, 1995), and under

water-limited conditions, as would be the case during the north African summer, trees are known to reduce leaf area (Weih *et al.*, 2011). While the overall moisture content during the twelve-month study period did not exhibit a difference between summer and winter months, and was not found to influence *P. fragrans* growth during the CCA analysis, it appears that the original, water-availability-determined growth cycle still occurs for *P. fragrans* growing in Ireland.

Some variation in light availability from month to month at the twelve sites was recorded. This was likely to be due to differing amounts of incident sunlight, as it was not feasible to monitor the available light levels on uniformly cloud-free days across the twelve-month period. Outside of this influence, the general trend was that available light levels were lowest during the summer months in both the shaded *P. fragrans* study sites and in the Cloyne ZDWS. During July to September, actual light levels in the shaded *P. fragrans* sites and the [+ willow + cover] ZDWS in Cloyne were similar (approximately 20 µmol m^{-2} s^{-1}).

As *P. fragrans* is known to be a shade-tolerant species (Devlin, 2014), this indicates that the available light reaching ground level in the ZDWS during the late summer is unlikely to have a detrimental effect on the percentage cover of *P. fragrans.* In contrast, a significant negative correlation was found between light availability and shoot density. Furthermore, the CCA analysis showed that plant dry weight exhibited a negative association with increasing light level. These results indicate that, in the presence of high light, the *P. fragrans* response is to produce more shoots per unit area, but to decrease the size i.e. dry weight, of individual shoots. Similar results were recorded by Huffman *et al.* (1994) for the rhizomatous species

Gaultheria shallon Pursh, where under 100% light intensity, the biomass was reduced compared to the optimum light intensity of 70%.

In general, the sand content of the soils where *P. fragrans* grew was high. Sites at Donoughmore, Glounthaune, Lee Road and Macroom had very high sand contents of greater than 80%. The twelve sites studied all had a clay content of less than 2%. Sites with clay contents greater than 13% are generally classified as low permeability in Ireland (Swartz *et al.*, 2003). The high sand content and very low clay content indicates that *P. fragrans* does not naturally colonise sites of low permeability. As ZDWS are generally constructed in areas where the subsoil has a high clay content and low permeability, it is likely that *P. fragrans* would not thrive in the soils typical of a ZDWS. Where *P. fragrans* was found in sites susceptible to occasional water inundation from an adjacent river, such as the sites at Cooperage, Lee Road and Atlantic Pond, the high sand content would allow for free-draining soil conditions. *P. fragrans* is known to favour humus-rich soil with a large amount of organic matter (Tys *et al.*, 2015) and in this trial rhizome length was found to be significantly positively correlated with organic matter percentage.

Evapotranspiration trial

Evapotranspiration was highest from the primary wastewater lysimeters, followed by the tertiary wastewater then rainwater lysimeters, and peaked during the summer months. This indicates the potential suitability for use of this species in a ZDWS receiving primary or tertiary wastewater. The increased evapotranspiration from the lysimeters receiving wastewater, compared to the lysimeters that received rainwater, was likely due to the increased availability of nutrients. However, the

amount of evapotranspiration that occurred during the winter months was very similar to the evaporation from the unplanted lysimeters and the reference evapotranspiration. Evaporation from ZDWS during the winter in Ireland has been found to be very close to the reference evapotranspiration (Curneen and Gill, 2015). The purpose of using *P. fragrans* in a ZDWS was to increase winter evapotranspiration above reference evapotranspiration or evapotranspiration from bare soil, but this did not occur during this trial. Thus, the benefits of using *P. fragrans* to increase winter evapotranspiration are negligible, if any. While the evapotranspiration rates from the *P. fragrans* lysimeters peaked during the summer months, this is the time period when willow evapotranspiration is highest. The annual k_c values for *P. fragrans* recorded during this trial were close to 1.0 indicating that the overall contribution of *P. fragrans* to annual evapotranspiration was limited.

Waterlogging trial

No significant differences in the aboveground biomass between the four treatments were found. However, the significant differences in belowground biomass showed that *P. fragrans* is not tolerant of waterlogged conditions. Belowground biomass and survival decreased with increasing duration of saturated soil conditions. This agrees with the findings of the site characterisation, where the sites were found to have very low clay contents, indicating good drainage. Furthermore, no signs of waterlogging were found at any of the site visits.

The F_v/F_m ratio is a commonly used measure to determine the effects of environmental stresses on plants (Maxwell and Johnson, 2000). Changes in

chlorophyll fluorescence can be used to study the effects of waterlogging on plants (Ahmed *et al.*, 2002). This ratio has previously been used to assess the effects of waterlogging on mung bean (Ahmed *et al.*, 2002) and alfalfa (Smethurst and Shabala, 2003). In this trial, differences in F_v/F_m values between waterlogged and control plants began to emerge after just two days of the waterlogged treatment, indicating that *P. fragrans* cannot tolerate even short-duration waterlogging events.

This waterlogging sensitivity would raise concerns over the suitability of the plant species for use in ZDWS. To allow for *P. fragrans* survival in a ZDWS setting the water level would have to be carefully controlled to ensure that the top layer of soil does not become waterlogged. According to the Danish guidelines, ZDWS should be designed so that the water level does not breach the surface of the soil. However, Curneen and Gill (2015) noted that the water levels are likely to be above the surface of the soil in a ZDWS in Ireland at least 55 days per year. In the presence of a covered ZDWS, along with subsurface distribution of wastewater the waterlogging on the surface of the soil could be limited. This may facilitate the use of *P. fragrans* as a winter-active species. However, as the purpose of a covered ZDWS is to maximise wastewater application, it is likely that waterlogged conditions would occasionally exist. As short duration waterlogging was shown to affect both belowground biomass and survival negatively, the use of *P. fragrans* in a ZDWS setting does not appear viable.

Intercropping trial

Intercropping willow with *P. fragrans* did not affect final height or diameter of the willow trees. However, intercropping did reduce final willow shoot biomass and

root biomass. As willow biomass is related to evapotranspiration (Curneen and Gill, 2014), a reduction in willow yield would reduce overall evapotranspiration levels from the willow crop. Intercropping willows with other species has previously been carried out with mixed success. The results recorded here are similar to the results of Wieshammer *et al.* (2007) who found that intercropping *S. caprea* with *Arabidopsis halleri* led to a reduction in *S. caprea* leaf biomass. Keeping the willow crop clear of weeds during the first year is important for willow establishment and yield (Sage, 1999; Caslin *et al*., 2010). It appears that the presence of *P. fragrans* during the establishment phase of the willows in this trial had a similar effect to that of high levels of weeds in willow SRC.

The presence of the willow also reduced the aboveground and belowground *P. fragrans* biomass. While there was no significant difference in percentage cover, monocrop percentage cover was approximately 50% greater than intercrop percentage cover. Competition between both species for water and nutrients was likely to have occurred in this trial given the small size of the pots. In the field, the effects of this competition may be reduced given the larger volume of soil that can be used for nutrient and water uptake. In addition, allelopathic effects of willow on *P. fragrans* may have occurred as *S. alba* leaf aqueous extracts have been shown to negatively affect the germination of *Triticum aestivum* L. (Abdel-Latif *et al*., 2015).

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Chapter 5 The influence of coppice management regime and soil type on the performance of zero-discharge willow wastewater treatment systems

5.1 Abstract

Willow evapotranspiration rates and crop coefficients for the Irish climate were recorded across two different coppice management regimes (coppiced and non-coppiced), and four different soil types (5%, 10%, 15% and 30% clay). Seasonal willow evapotranspiration rates varied from 640 to 1200 mm over two years. Willows managed in an annual coppice management regime, as would be the case under the conditions of a polytunnel system, removed 114 – 190 mm less wastewater through evapotranspiration than trees that were not coppiced. This was due to the larger size of the non-coppiced trees. Soil type had a limited effect on evapotranspiration, and was more important for trees growing in a longer duration non-coppiced cycle. Seasonal crop factors varied from 2.3 to 3.6. For non-coppiced trees, those growing in 10% clay had the lowest seasonal evapotranspiration, which was likely due to lower nutrient levels in the soil affecting growth. Biomass production during both years of annual coppicing was similar, indicating that a short duration of annual coppicing may not negatively impact growth. Nutrient attenuation of the wastewater by the system was quite high, and similar to the results of field studies reported elsewhere. While nutrient concentration was higher in the trees undergoing the annual coppicing cycle, nutrient content was higher in the non-coppiced trees, indicating the importance of the larger biomass of the non-coppiced trees in removing larger amounts of nutrients during harvest.

5.2 Introduction

5.2.1 Suitability of willows for wastewater treatment

Willows are frequently used in the treatment of wastewater due to high rates of nutrient removal through harvesting (Amofah *et al*., 2012), increase in biomass production in response to nutrient application (Perttu and Kowalik, 1997; Larsson *et al.*, 2003) and the ability of willow to tolerate flooded conditions (Jackson and Attwood, 1996; Kuzovkina *et al*., 2004). Furthermore, the evapotranspiration rate of willow SRC is very high, and generally exceeds that of other agricultural crops or tree species grown in the UK (Hall *et al*., 1998). The high evapotranspiration rate of willows has led to the use of willows in ZDWS (Gregersen and Brix, 2001).

Knowledge of the evapotranspiration rate and crop factor of willow allows for the appropriate sizing of ZDWS (Ministry of Environment and Energy, 2003). To date, few studies have been carried out on evapotranspiration rates and crop factors for willows in Ireland. Willow evapotranspiration in Ireland has been recorded as approximately 600 – 1100 mm annually (Curneen and Gill, 2014, 2015). In Ireland, growing season crop factor values as high as 5.1 have been recorded for willow trees receiving primary wastewater (Curneen and Gill, 2014). Maximum k_c values of 2.5 have been recorded in the UK for willow trees receiving secondary wastewater (Sugiura, 2009).

5.2.2 The influence of willow coppice management regime on wastewater removal and treatment

In an attempt to reduce rainfall entry into ZDWS, and to increase the amount of wastewater that can be applied on a unit area basis, the use of a polytunnel-

covered wetland was trialled and is described in Chapter 3. As part of a polytunnel-covered system, annual coppicing would need to take place as willow growth above 2.5 m would impact on the structural integrity of the polytunnel.

Trees undergoing annual coppicing cycles have lower yields than those with longer harvest cycles (Szczukowski *et al.*, 2002, 2005; Stolarski *et al.*, 2008). Different willow species such as *Salix alba* L., *S. purpurea* L. and *S. dasycladoss* Wimm. have a range of suitability to annual coppicing, and well-adapted clones can maintain productivity through ten annual harvests (Kopp *et al.*, 2001). Further study is required to determine changes in evapotranspiration and survival between annually coppiced trees and trees undergoing a longer coppicing cycle or a non-coppiced cycle.

For willow SRC, shorter coppice cycles led to increased stem nutrient concentrations in the harvested biomass (Ericsson, 1994). Despite enhanced nutrient removal through biomass harvesting in SRC systems, effluent application can increase soil nitrogen and phosphorus content to levels above those which are optimum for crop growth (Aronsson and Perttu, 2001; Paranychianakis *et al.*, 2006; Tzanakakis *et al.*, 2011). As ZDWS are designed on the basis of ability to remove water volume, rather than to retain/remove nutrients, the build-up of nitrogen and phosphorus within the system should be explored.

5.2.3 The influence of soil type on wastewater removal and treatment

Low-permeability sites have been found to contain at least 13% clay (Swartz *et al.*, 2003). As ZDWS are generally located in areas of low subsoil permeability (Gregersen and Brix, 2001), it is likely that high clay soils will be used. Soil type is a controlling factor on willow yield, with willow yield in Ireland varying from 7.5 to 10.5 odt ha^{-1} yr^{-1}, depending on soil type (van den Broek *et al.*, 1997). Medium to heavy clay loams are regarded as being ideal for willow growth due to high nutrient levels in clay soils (Labrecque and Teodorescu, 2001; Caslin *et al.*, 2010). Sizing of ZDWS should take into account any differences in yield, and hence evapotranspiration, that may occur as a result of differences in soil type.

In addition to affecting willow yield, soil type can also affect nutrient removal through willow harvesting. Higher nutrient removal from clay sites in comparison to sandy sites have been attributed to differences in yield between the clay and sandy sites (Aronsson and Bergström, 2001; Rytter, 2001; Dimitriou and Aronsson, 2004).

5.2.4 Objectives

The objectives of the research described in this chapter are:

(a) to investigate the impact of two different coppice management regimes (coppiced and non-coppiced) on the evapotranspiration rate, growth rate and nutrient balance of a series of zero-discharge willow lysimeters;

(b) to investigate the impact of a gradient in clay content on the evapotranspiration rate, growth rate and nutrient balance of a series of zero-discharge willow lysimeters.

5.3 Materials and Methods

5.3.1 Water balance measurement

Lysimeters, previously installed in the School of BEES glasshouse area in UCC, were used in this trial. Lysimeters consisted of plastic water butts (190 L) in the shape of a truncated cone (Figure 5.1). Each lysimeter was placed on a 0.3 m high plastic or concrete block base and filled to a height of 0.7 m with soil. A tap at the base of the lysimeter allowed for sampling of the soil water. One *Salix viminalis* L. willow cutting (Alba Trees Ltd., East Lothian, Scotland) was planted in the centre of each lysimeter in 2007. Due to previous research in the School of BEES, the lysimeters used in this work were interspersed among other lysimeters containing *Eucalyptus gunnii* Hook. f.

There were two factors in this trial: management regime and soil type. For management regime, two different coppicing regimes were followed: coppiced and non-coppiced. This coppice management system was started in 2011 and coppiced trees were cut back during the winter of each year. Non-coppiced trees were not cut back. For soil type, each lysimeter had previously been filled with one of four soil types, namely topsoil mixed with sand to give a final clay content of approximately 5%, 10%, 15% or 30% clay. Soil analysis (Table 5.1) was carried out following the methods of section 3.3.1. In total, 37 lysimeters were used in this trial: 5% clay and coppiced (n = 4), 10% clay and coppiced (n = 5), 15% clay and coppiced (n = 5), 30% clay and coppiced (n = 3), 5% clay and non-coppiced (n = 5), 10% clay and non-coppiced (n = 7), 15% clay and non-coppiced (n = 3), 30% clay and non-coppiced (n = 5).

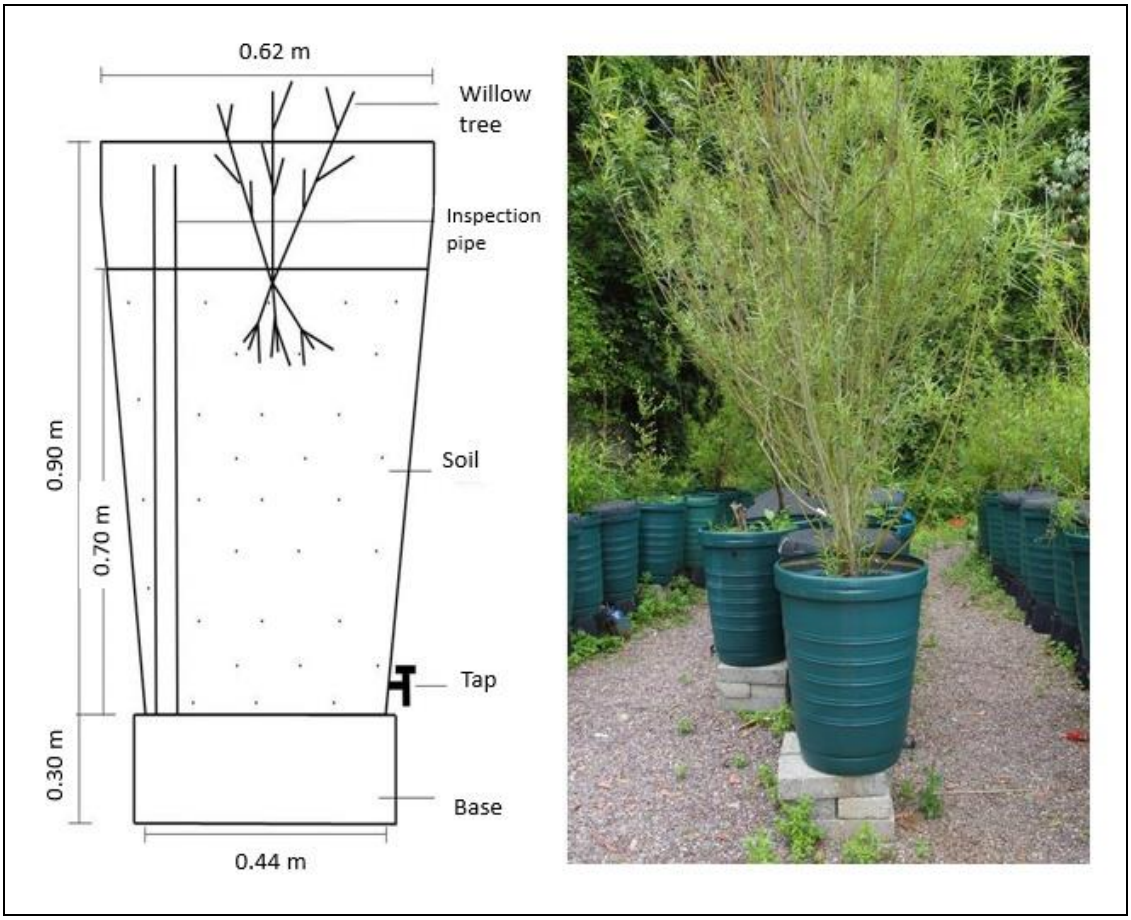


Figure 5.1 Lysimeter schematic (left) and image from experimental site (right).

Table 5.1 Soil characteristics for each of the four soil types.

Parameter	5% clay	10% clay	15% clay	30% clay
pH	6.9	6.6	7.0	7.4
Electrical conductivity$_{1:5}$ (µS cm^{-1})	124.0	106.2	122.1	146.3
Organic matter content (% LOI)	3.6	2.6	3.5	5.8

At the start of the experiment, an inspection pipe was installed in each lysimeter to allow measurement of water volume and calculation of the lysimeter water balance (Figure 5.1). A vertical soil core was dug to the base of each lysimeter using a 5 cm diameter soil core auger. A 38.1 mm diameter PVC waste pipe, 0.8 m long, was placed into the cored hole. At the end of each pipe, eight 5 mm diameter holes

were drilled. The holes allowed water from the surrounding soil to enter the pipe from the end. Each lysimeter was calibrated before the start of the trial by adding known quantities of water and allowing the water level in the inspection pipe to settle. The height of the water in the inspection pipe was then measured. Calibration curves were constructed for each lysimeter allowing the volume of water in the lysimeter to be determined, for any given height of water in the inspection pipe. Calibration was carried out to a height approximately 10 – 15 cm above the level of the soil. As the water level rise in the soil was much higher than in the air (for the same volume of water added), a pattern of two different curves resulted. The first line represented the height-volume relationship in the soil phase, and the second line represented the height-volume relationship for the air phase (Figure 5.2). To calculate the water used on a week-to-week basis, the calibration equation to be used depended on the height measured in the inspection pipe.

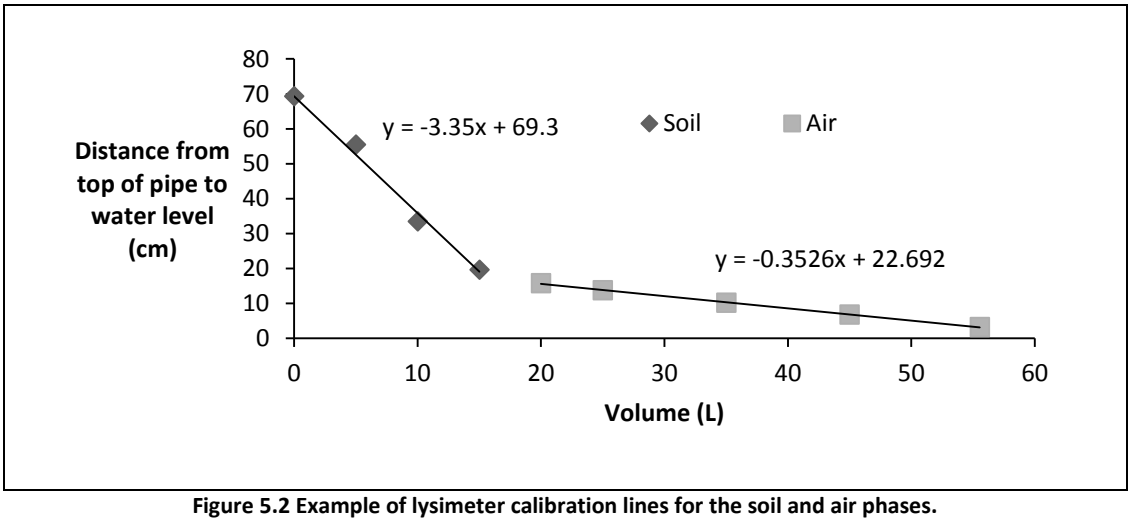


Figure 5.2 Example of lysimeter calibration lines for the soil and air phases.

The water balance of the lysimeters was described by rearranging equation 2.2 to give:

$$ET = \pm \Delta S + P + Q$$ **Equation 5.1**

where ET = evapotranspiration, ΔS = change in water storage, P = precipitation and Q = wastewater irrigation. The change in water storage was calculated using the calibration curves. Wastewater irrigation was carried out weekly using synthetic domestic primary wastewater (section 4.3.2). Precipitation was measured using a ClimeMET professional manual rain gauge (Skyview Systems Ltd., Sudbury, UK) located in the centre of the site. Lysimeters were irrigated weekly from the start of May to the end of September during 2014 and 2015. Irrigation was carried out using a watering can, so that wastewater was added to return the level in the inspection pipe to the level of the top of the soil.

5.3.1.1 Climate measurements

Reference evapotranspiration (ET_o) was calculated using a Class A evaporation pan located in the centre of the site, except for a period at the start of May 2014 while the fixed point gauge was being built. During this period, ET_o was calculated using the Hargreaves ET_o equation:

$$ET_o = 0.0023(T_{mean} + 17.8)(T_{max} - T_{min})^{0.5}R_a$$ **Equation 5.2**

where ET_o and R_a were expressed in units of mm day^{-1}, R_a = extraterrestrial radiation, T_{mean} = mean daily temperature, T_{max} = maximum daily temperature, T_{min} = minimum daily temperature. R_a was estimated following the guidelines of Allen

(1998) using GSR (MJ m^{-2}) data from Roche's Point meteorological station (the nearest meteorological station to record these data, approximately 15 km from the trial site) according to:

$$R_a \approx GSR/0.5 \quad \textbf{Equation 5.3}$$

R_a (mm day^{-1}) was calculated using the equation of Allen (1998):

$$R_a \text{ (mm day}^{-1}) = 0.408 \times R_a \text{ (MJ m}^{-2} \text{ day}^{-1}) \quad \textbf{Equation 5.4}$$

After this initial period, ET_o (mm) was calculated according to:

$$E_{pan}k_{pan} = ET_o \quad \textbf{Equation 5.5}$$

where E_{pan} = pan evaporation (mm), and k_{pan} = pan factor. A pan factor of 1.1 was selected by following the guidelines of Allen *et al.* (1998) and by comparing calculated ET_o values to ET_o values from the Met Éireann site at Cork Airport (approximately 6 km from the site). Evaporation pan measurements were taken daily between 9 and 10 am. Evapotranspiration values were compared to reference evapotranspiration values to calculate the crop factor (k_c) according to:

$$k_c = ET_c/ET_o \quad \textbf{Equation 5.6}$$

Temperature and relative humidity were measured on site using a HOBO Pro V2 data logger (Onset Computer Corporation, Massachusetts, USA).

5.3.2 Tree growth measurements

For the coppiced trees, height was measured from the point of emergence from the stump to the apex of the leading shoot at the end of the growing season. For the non-coppiced trees, the height from the top of the tallest stem to the soil surface in the lysimeter was measured at the end of the growing season. Leading shoot diameter of the coppiced trees was measured at 0.3 m from the point of shoot emergence using a digital calipers (Guidi, 2008). The diameter of the main shoot on the non-coppiced trees was measured at the end of each growing season at a height of 0.3 m above the soil. Shoot number of the coppiced trees was recorded at the end of the trial.

Stem volume index (SVI) was calculated for both coppiced and non-coppiced trees at the end of each growing season. SVI (dm^3) was calculated according to the equation of Guidi (2008):

$$SVI = hd_{0.3}^2 \qquad \textbf{Equation 5.7}$$

where h = height of stem (cm) and d = diameter (mm) of main stem at 0.3 m above the soil surface in the case of non-coppiced trees, and 0.3 m from the point of emergence from the stump in the case of coppiced trees. For the non-coppiced, trees the index was applied to the main stem. For the coppiced trees, the volume index of each stem per tree was calculated and the values summed to give an index for each tree (Herve and Ceulemans, 1996).

Stem basal area (SBA)(cm^2) was calculated following the method of Proe *et al.* (1999). For each coppiced tree, diameter was measured with a digital calipers at the

point of emergence from the stump and converted to an area assuming a circular cross-section, with individual stems summed to give tree stem basal area (Martin and Stephens, 2006a). For the non-coppiced trees, tree SBA was taken as the basal area of the main stem at the point of emergence from the soil (assuming circular cross-section) (Proe *et al.*, 1999).

Leaf area index (LAI) was calculated for the coppiced trees through allometric measurements and relationships following the methods of Martin and Stephens (2006a, 2006b). In late July each year, a sample of thirty leaves of different sizes was taken and the length and width of each leaf was measured using a digital calipers. The actual area of each leaf was then calculated using Image J image analysis software (National Institutes of Health, Maryland, USA). A linear relationship was derived between length and width, and actual area according to:

$$A = b(LW) \qquad \textbf{Equation 5.8}$$

where A = actual area measured using Image J (cm^2), b = coefficient for each soil type (5% clay: b = 0.725; 10% clay: b = 0.744; 15% clay: b = 0.728; 30% clay: b = 0.719), L = length (cm) and W = width (cm). To calculate the stem leaf area for each soil type, six stems, across a range of stem diameters, were selected from each soil type. The stem leaf area was calculated as the product of the number of leaves per stem and the average leaf area. The average leaf area was calculated by measuring the length and width of every tenth leaf on the stem, and converting that to actual average leaf area using equation 5.8. Simple linear regression was used to define the relationship between stem leaf area and SBA. Once SBA for each individual stem was known, tree leaf area could then be calculated. As SBA on all stems was

calculated at the end of the growing season only, tree leaf area was calculated as the maximum tree leaf area that would have occurred during the growing season.

To calculate maximum tree LAI, maximum canopy diameter was first calculated. The canopy diameter was measured in a North-South and East-West direction at the end of the growing season. The average of these two measurements was used to calculate the projected ground canopy cover area for each tree, assuming this was a circle. Maximum LAI was then calculated by dividing the maximum tree leaf area by the projected ground canopy cover area. Maximum LAI was not calculated for the non-coppiced trees due to the different growth form (many second, third and fourth order stems, whereas coppiced trees had first order stems only).

Water use efficiency (WUE) for the coppiced trees was measured as the ratio of dried harvestable stem biomass (g) to water used (L) during the growing season (Bonneau, 2004). For the coppiced trees, stems were cut back to the point of emergence from the stump. For the non-coppiced trees at the end of 2015, trees were harvested to the level of the top of the lysimeter. Fresh weight of each tree was recorded. Subsamples (approximately 10% of the total weight harvested) were oven dried at 60 °C to a constant weight.

5.3.3 Nutrient analysis

5.3.3.1 Wastewater sampling and analysis

The soil water within the lysimeters was tested twice for TN and TP over the course of the trial: once during the winter of 2014 and once during the summer of 2015. Wastewater was sampled by opening the tap at the base each lysimeter. TP was measured by sample digestion followed by colorimetric analysis using the ascorbic

acid method, in which all forms of phosphorus are oxidised to orthophosphate (American Public Health Association *et al.*, 1989). Fifty mL of sample, standard or water (blank) was placed in an acid-washed Duran® bottle (250 mL). One ml of 10.8 N sulphuric acid and 0.44 g of ammonium persulphate were added. Samples were then autoclaved for thirty minutes (121 °C, 15 p.s.i.). Once cool, 8 mL of a combined reagent was then added to each sample. The combined reagent consisted of 1.4 N sulphuric acid, potassium antimonyl tartrate, ammonium molybdate and ascorbic acid (American Public Health Association *et al.*, 1989). After ten minutes, the absorbance of each sample was measured at 880 nm using a Genesys 10 UV-vis spectrophotometer (Thermo Electron Scientific Instruments LLC, Madison, WI, USA). A calibration curve was prepared using anhydrous potassium dihydrogen phosphate. At least one phosphate standard was tested with each set of samples.

Total Nitrogen was measured using the method of Gross *et al.* (1999), whereby all forms of nitrogen are oxidised to nitrate. A 10 mL aliquot of each sample was placed in an acid-washed Duran® bottle (250 mL), along with 5 mL of 0.075 N sodium hydroxide and 0.1 g potassium persulphate. Samples were mixed by inverting the bottle twice. Bottles were autoclaved for thirty minutes at 121 °C. Borate buffer solution was prepared by making 61.8 g boric acid and 8 g sodium hydroxide up to 1,000 mL with distilled water. Buffer solution (1 mL) was added to each sample and samples were mixed by inverting the bottle twice. Samples were centrifuged at 14,000 x g for five minutes. The supernatant was then analysed spectrophotometrically for nitrate using method 4500-NO_3^- B (American Public Health Association *et al.*, 1989). Absorbance was measured at 220 nm for nitrate

and 275 nm to correct for interference due to dissolved organic matter. For samples and standards, the absorbance due to nitrate was determined by:

$$A_{(nitrate)} = A_{220} - 2(A_{275}) \quad \textbf{Equation 5.9}$$

where $A_{nitrate}$ = absorbance due to nitrate, A_{220} absorbance at 220 nm and A_{275} = absorbance at 275 nm. A calibration curve was prepared using oven-dry potassium nitrate.

5.3.3.2 Soil and plant biomass nutrient analysis

Soil was sampled at the start and end of the trial and frozen until analysed. Two soil cores were sampled from each lysimeter using a 5 cm core auger to a depth of 20 cm. Samples were homogenised, air dried and sieved through a 2 mm sieve. TN and TP analyses were carried out in the Aquatic Services Unit of the Environmental Research Institute, UCC. Plant biomass was sampled at the end of each growing season for the trees undergoing the annual coppicing cycle, and at the end of the second growing season for the non-coppiced trees. Three segments were taken at three levels of the main stem (bottom, middle and top)(Labrecque and Teodorescu, 2003). Segments from the three levels were oven dried at 60°C, chopped and ground together to form a composite sample (Adegbidi *et al.*, 2001).

Soil and tree samples were analysed for TN and TP by the Aquatic Services Unit. Samples were digested at 420 °C using concentrated sulphuric acid and one Kjeltab and followed by TKN analysis using a Lachat Quik-Chem 8000 Flow Injection analyser (FIA) or TP analysis using a FIA star 5010 Analyser. Tree nutrient content was expressed as concentration (g kg^{-1}) multiplied by the dry weight (kg) of the biomass harvested.

5.3.4 Statistical analysis

Variables were assessed for normality and homogeneity of variance before or after transformation. Evapotranspiration, biomass, height, diameter, SBA and shoot number data were normalised by square root transformation. Where possible, data were subjected to parametric two-way ANOVA to test the effects of soil type and coppice management regime. Multiple comparison tests were conducted using the Tukey test. Within the coppiced treatment, maximum tree leaf area, maximum LAI, shoot number and WUE data were subjected to a one-way ANOVA. Multiple comparison tests were conducted using the Tukey test. The change in soil TN and TP data (within group) from the start to the finish of the trial was non-normal and was analysed using Wilcoxon's Paired Sample test. Descriptive statistics are presented as non-transformed values. Statistical analyses were carried out using SPSSv20.

5.4 Results

5.4.1 Water balance

The growing season (May – September) of 2015 was much wetter than that of 2014, with a total of 519 mm of rain falling during the growing season in 2015, compared to a total of 292 mm for the same period in 2014 (Figure 5.3). Reference evapotranspiration was higher during the growing season of 2014 (338 mm), compared to 2015 (315 mm). The average temperature during July, August and

September was slightly higher in 2014 (mean value of 16.4 °C) than in 2015 (mean value of 14.2 °C).

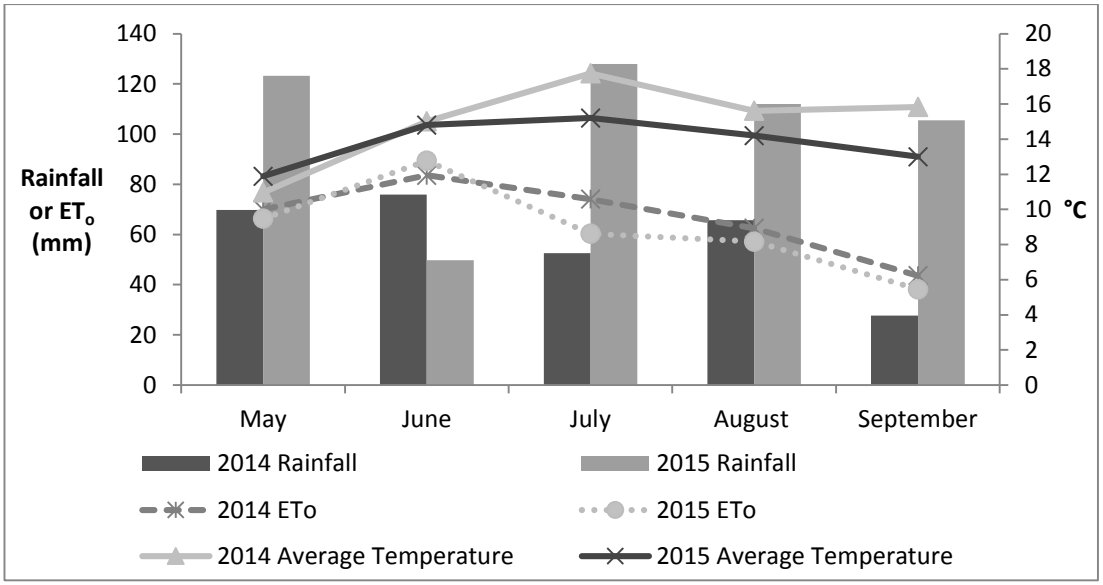


Figure 5.3 Average temperature, reference evapotranspiration and rainfall during the experimental periods of 2014 and 2015.

In both years daily evapotranspiration rates were highest during mid-summer, and lowest at the start and end of the summer (Figure 5.4). In general, daily evapotranspiration rates of the non-coppiced trees were higher than the coppiced trees. During 2014, peak evapotranspiration rates for the coppiced trees occurred towards the end of July, with values ranging from 8.8 mm day^{-1} (30% clay) to 10.8 mm day^{-1} (5% clay). Peak values for the non-coppiced trees were recorded during June, ranging from 10.7 mm day^{-1} (10% clay) to 11.5 mm day^{-1} (30% clay).

In 2015, peak evapotranspiration for the coppiced trees occurred during June, ranging from 7.1 mm day^{-1} (30% clay) to 11.1 mm day^{-1} (5% clay). For the non-coppiced trees, peak values also occurred during June, ranging from 9.7 mm day^{-1} (30% clay) to 11.7mm day^{-1} (15% clay).

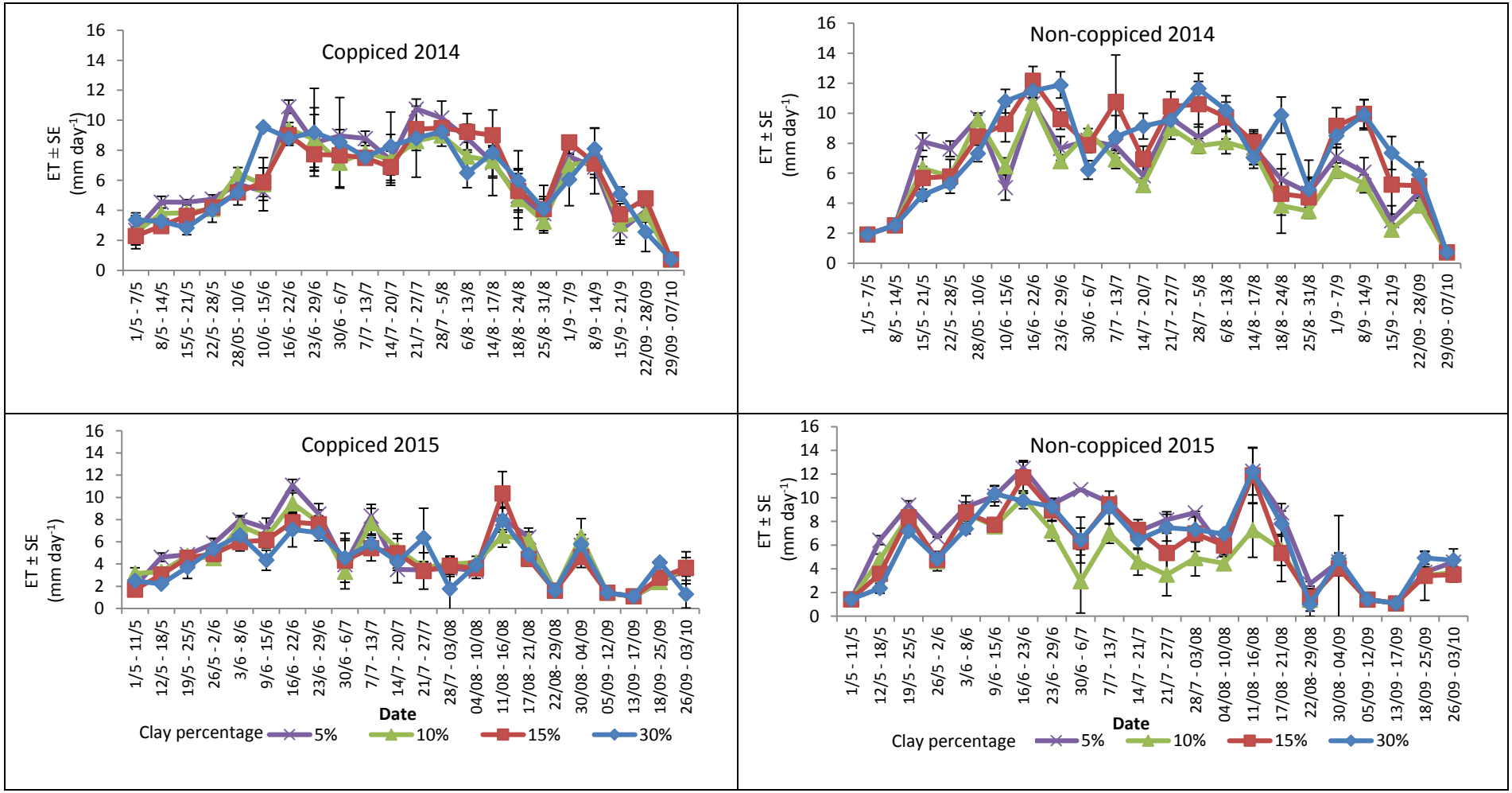
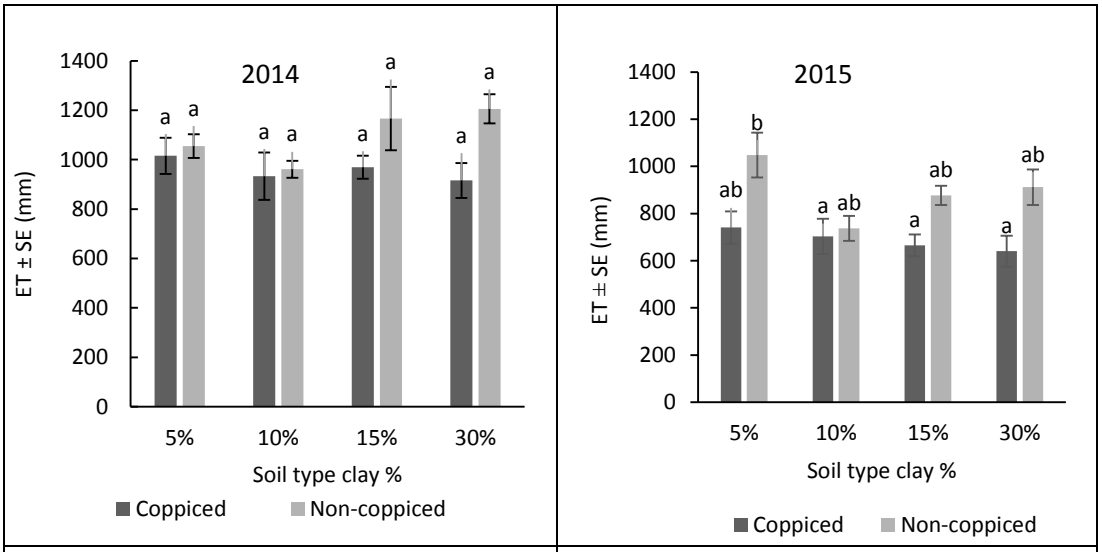


Figure 5.4 Mean ± SE daily ET values during the growing seasons of 2014 and 2015.

In 2014, non-coppiced trees (1076.4 mm) had significantly higher seasonal ET values than did coppiced trees (960.0 mm)(Figure 5.5). There was no significant difference in evapotranspiration between the four soil types and there was no significant [soil type x management] interaction. A similar trend occurred in 2015. Non-coppiced trees (879.3 mm) had significantly higher seasonal evapotranspiration values than did coppiced trees (689.6 mm)(Figure 5.5). There was no significant difference in evapotranspiration between the four soil types and there was no significant [soil type x management] interaction. In both 2014 and 2015, coppiced trees grown in 5% clay had the highest seasonal evapotranspiration. For the non-coppiced trees, in 2014, trees grown in 30% clay had the highest evapotranspiration while in 2015, trees grown in 5% clay had the highest seasonal evapotranspiration.



ANOVA	df	F	P
Soil type	3	1.60	>0.05
Management	1	8.34	**<0.05**
Soil type x management	3	1.60	>0.05

ANOVA	df	F	p
Soil type	**3**	2.22	>0.05
Management	**1**	16.57	**<0.01**
Soil type x management	3	1.60	>0.05

Figure 5.5 Mean ± SE total evapotranspiration values for 2014 and 2015 growing seasons. Any samples within year which share a common letter are not significantly different using the Tukey test (p > 0.05).

The k_c values were low at the start of the growing season, but much more variable in the mid to late summer (Figure 5.6). In general, peak k_c values for coppiced and non-coppiced trees occurred in September of both years. Crop factor values were generally higher in the non-coppiced trees. In September 2014, the highest k_c values for the coppiced trees ranged from 2.8 (30% clay) to 5.3 (15% clay). For the non-coppiced trees during the same month, the highest k_c values ranged from 4.2 (10% clay) to 6.5 (30% clay). In September 2015, peak k_c values for the coppiced trees ranged from 3.6 (15% clay) to 5.1 (10% clay), while for the non-coppiced trees, values ranged from 4.1 (15% clay) to 5.6 (30% clay).

Mean seasonal k_c values in 2014 were higher in the non-coppiced trees compared to the coppiced trees, with the exception of non-coppiced trees growing in 10% clay which showed similar values to the coppiced trees (Table 5.2). In the non-coppiced trees, values varied from 2.8 (15% clay) to 3.6 (30% clay). For coppiced trees, values varied from 2.7 (30% clay) to 3.0 (5% clay). In 2015, a similar trend to 2014 emerged, but k_c values were slightly lower across all eight treatments. For non-coppiced trees, values varied from 2.3 (10% clay) to 3.3 (5% clay). For coppiced trees, values varied from 2.0 (30% clay) to 2.4 (5% clay).

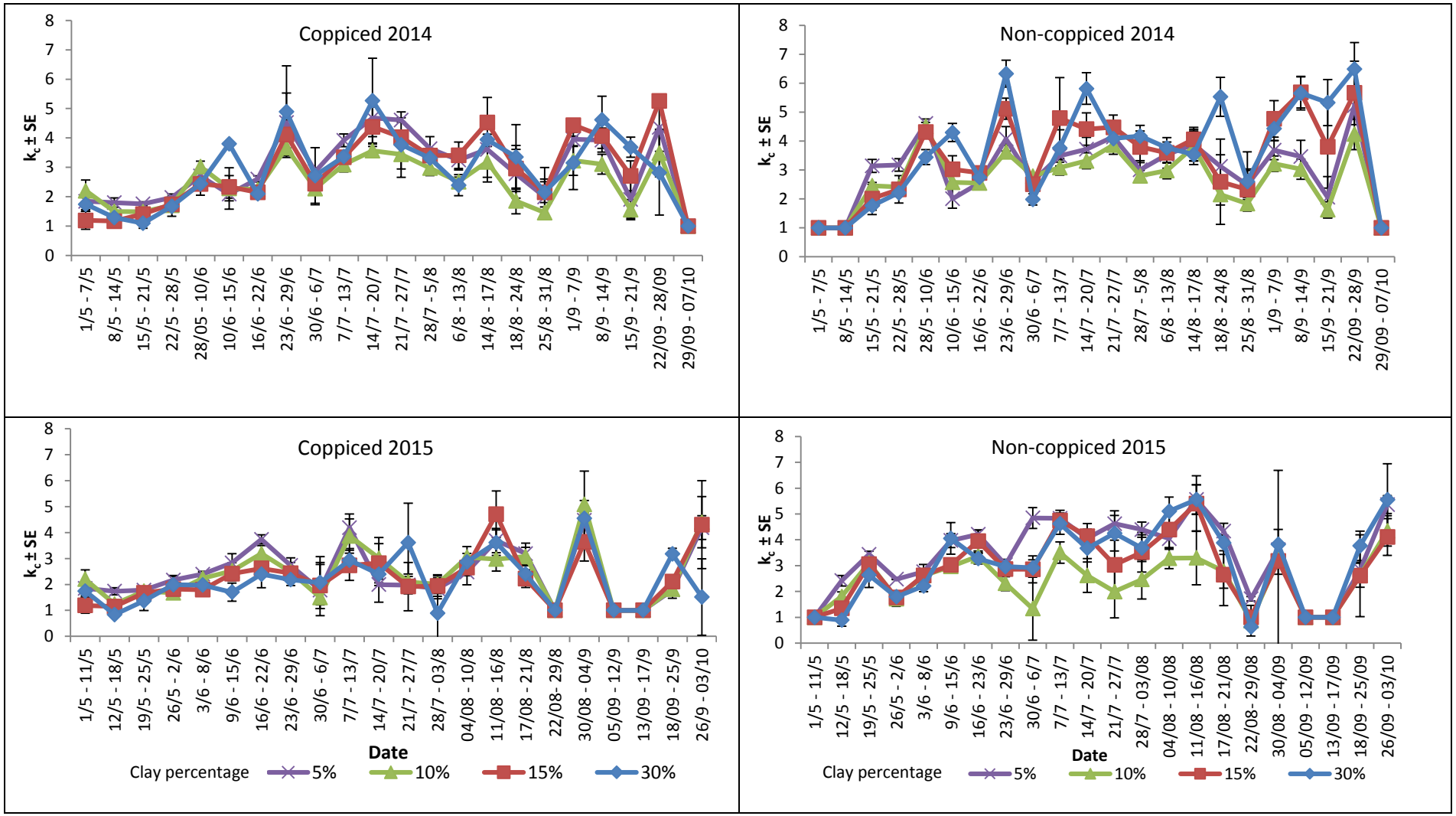


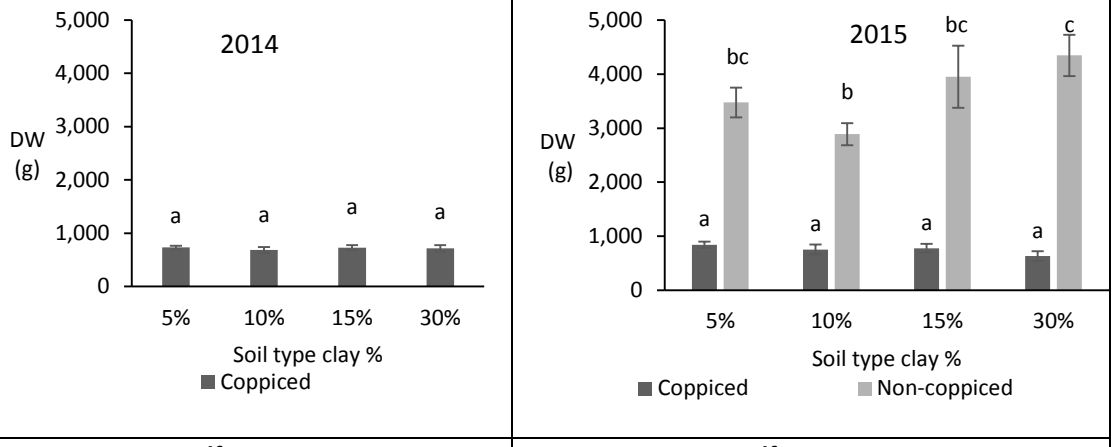
Figure 5.6 Mean ± SE k_c values during the growing seasons of 2014 and 2015.

Table 5.2 Mean ± SE seasonal k_c values for 2014 and 2015.

Year	Soil type clay %	Coppiced	Non-coppiced
2014	5%	3.0 ± 0.22	3.1 ± 0.14
	10%	2.8 ± 0.28	2.8 ± 0.10
	15%	2.9 ± 0.14	3.5 ± 0.38
	30%	2.7 ± 0.21	3.6 ± 0.17
2015	5%	2.4 ± 0.22	3.3 ± 0.30
	10%	2.2 ± 0.24	2.3 ± 0.17
	15%	2.1 ± 0.15	2.8 ± 0.13
	30%	2.0 ± 0.21	2.9 ± 0.24

5.4.2 Growth

Soil type did not have a significant effect on dry weight biomass of the coppiced trees in 2014 (Figure 5.7). Biomass varied from an average of 687 g tree^{-1} for trees growing in 10% clay to 733 g tree^{-1} for trees growing in 5% clay. At the end of 2015 non-coppiced trees had a significantly larger biomass than did the coppiced trees. There was also a significant [soil type x management] interaction. Soil type did not have a significant effect on shoot biomass in 2015. Non-coppiced biomass varied from an average of 2.89 kg tree^{-1} growing in 10% clay to 4.35 kg tree^{-1} growing in 30% clay. For coppiced trees, growth varied from an average of 632 g tree^{-1} in 30% clay to 840 g tree^{-1} in 5% clay.



ANOVA	df	F	P
Soil type	3	0.24	>0.05
Management	-	-	-
Soil x management	-	-	-

ANOVA	df	F	P
Soil type	3	2.55	>0.05
Management	1	468.47	**<0.01**
Soil x management	3	3.93	**<0.05**

Figure 5.7 Mean ± SE dry weight at end of 2014 and 2015. Any samples within a year which share a common letter are not significantly different using the Tukey test (p > 0.05).

In 2014, the effect of management regime on height was significant and there was also a significant [soil type x management] interaction (Table 5.3). Soil type did not have a significant effect on tree height. Non-coppiced trees were larger than coppiced trees across all soil types. For non-coppiced trees, those growing in 30% clay were tallest, reaching an average height of 413 cm, while non-coppiced trees growing in 10% clay were the smallest, reaching a final height of 304 cm. A similar trend occurred in 2015. The effect of management regime was significant and there was also a significant [soil type x management] interaction, though soil type did not have a significant effect on plant height. For non-coppiced trees, those growing in 30% clay were tallest, reaching an average height of 424 cm. Non-coppiced trees growing in 10% clay were the smallest, reaching a final height of 313 cm.

Table 5.3 Mean ± SE height, diameter, SBA and SVI values in 2014 and 2015. Any samples within year which share a common letter are not significantly different using the Tukey test (p > 0.05).

Year	Management regime	Soil type clay %	Height (cm)	Diameter (mm)	SBA (cm^2)	SVI (dm^3)
2014	**Coppiced**	**5%**	224 ± 24 a	10.5 ± 0.6 a	23.1 ± 0.9 ab	2.84 ± 0.60 a
		10%	251 ± 9 a	10.5 ± 0.5 a	21.4 ± 1.7 a	2.64 ± 0.26 a
		15%	267 ± 17 a	12.0 ± 0.7 a	20.4 ± 1. 1 a	2.96 ± 0.22 a
		30%	224 ± 14 a	10.6 ± 0.7 a	23.4 ± 2.5 ab	2.93 ± 0.22 ab
2014	**Non-coppiced**	**5%**	307 ± 16 ab	40.1 ± 3.4 b	35.3 ± 4.9 ab	7.18 ± 0.60 b
		10%	304 ± 21 ab	34.7 ± 1.7 b	26.3 ± 2.7 ab	5.14 ± 0.57 ab
		15%	311 ± 34 ab	36.4 ± 6.7 b	44.1 ± 12.1 b	5.95 ± 3.43 ab
		30%	413 ± 37 b	37.9 ± 4.4 b	32.5 ± 1.0 ab	11.22 ± 1.84 c

2014	**ANOVA**		**F**	**P**	**F**	**P**	**F**	**P**	**F**	**p**
		Soil type	1.50	>0.05	0.18	>0.05	1.37	>0.05	5.04	**<0.01**
		Management	29.42	**<0.001**	233.80	**<0.001**	21.90	**<0.001**	54.67	**<0.001**
		Soil x management	3.10	**<0.05**	0.61	>0.05	1.87	>0.05	4.37	**<0.05**

Year	Management regime	Soil type clay %	Height (cm)	Diameter (mm)	SBA (cm^2)	SVI (dm^3)
2015	**Coppiced**	**5%**	183 ± 13.9 a	9.3 ± 0.7 a	28.0 ± 1.9 abc	2.39 ± 0.23 a
		10%	232 ± 8.2 ab	11.1 ± 0.7 a	23.1 ± 2.3 a	2.41 ± 0.27a
		15%	186 ± 17.4 ab	11.6 ± 0.5 a	21.8 ± 1.3 a	2.60 ± 0.36 a
		30%	191 ± 23.0 a	9.6 ± 1.0 a	22.1 ± 2.4 ab	2.02 ± 0.37 ab
2015	**Non-coppiced**	**5%**	318 ± 17.4 bc	42.9 ± 3.6 b	39.5 ± 5.7 bc	9.15 ± 1.04 cd
		10%	313 ± 21.1 b	37.4 ± 2.3 b	30.9 ± 2.7 abc	6.23 ± 0.59 abc
		15%	337 ± 25.2 bc	39.4 ± 7.2 b	37.4 ± 9.8 c	7.61 ± 4.39 bc
		30%	424 ± 35.5 c	40.1 ± 4.4 b	36.3 ± 2.5 abc	12.72 ± 1.97 d

2015	**ANOVA**		**F**	**P**	**F**	**P**	**F**	**P**	**F**	**p**
		Soil type	1.69	>0.05	0.09	>0.05	1.92	>0.05	3.58	**<0.05**
		Management	79.86	**<0.001**	270.76	**<0.001**	32.81	**<0.001**	86.25	**<0.001**
		Soil x management	4.03	**<0.05**	0.86	>0.05	1.87	>0.05	4.50	**<0.05**

Similar trends occurred in tree diameter. The diameters of the non-coppiced trees were significantly larger than those of coppiced trees in both 2014 and 2015 (Table 5.3). Soil type did not have a significant effect on diameter in either year and there was no significant [soil type x management] interaction. For coppiced trees, in both years, trees growing in 15% clay had the largest diameter. For non-coppiced trees, in both years, trees growing in 5% clay had the largest diameters.

In 2014, non-coppiced trees had significantly larger SBAs than coppiced trees (Table 5.3). For the coppiced trees, SBA varied from 20.4 cm^2 (15% clay) to 23.4 cm^2 (30% clay). In the non-coppiced trees, SBA varied from 26.3 cm^2 (10% clay) to 44.1 cm^2

(15% clay). Soil type did not have a significant effect and there was no [soil type x management] interaction. A similar trend occurred in 2015. Non-coppiced trees had a significantly larger SBA than coppiced trees. Soil type did not have a statistically significant effect on SBA and there was no [soil type x management] interaction.

Both coppice management regime and soil type had a significant effect on SVI and there was also a significant [soil type x management] interaction (Table 5.3). Non-coppiced trees had larger SVI than coppiced trees. For the coppiced trees, SVI was similar across all four soils, varying from 2.6 dm^3 (10% clay) to 2.9 dm^3 (15% and 30% clay) dm^3. Larger differences in SVI occurred between the non-coppiced tree populations, varying from 5.1 dm^3 (10% clay) to 11.2 dm^3 (30% clay). Similar trends occurred in 2015.

There were no statistically significant differences in shoot number, maximum tree leaf area, maximum LAI or WUE in the coppiced trees in 2014 (Table 5.4). In 2015, no significant differences were observed in these growth parameters, except for maximum tree leaf area. Coppiced trees growing in 5% clay had a maximum tree leaf area that was significantly higher than the maximum tree leaf area for trees growing in 10% or 15% clay soils. Trees growing in 30% clay exhibited a maximum tree leaf area which was not significantly different from those of trees grown in the other soil types. There was no difference in survival between coppiced and non-coppiced, with 100% of all trees surviving until the end of the trial (data not shown).

Table 5.4 Mean ± SE final shoot number, maximum tree leaf area, maximum LAI and WUE for the coppiced trees in 2014 and 2015. Any samples within year which share a common letter are not significantly different using the Tukey test (p > 0.05).

Year	Management	Soil type clay %	Shoot No.		Maximum tree leaf area (m^2)		Maximum LAI		WUE (g dm kg^{-1} water)	
2014	**Coppiced**	**5%**	60.5 ± 5.0 a		4.78 ± 0.22 a		3.47 ± 0.71 a		2.44 ± 0.24 a	
		10%	59.2 ± 6.7 a		3.65 ± 0.27 a		3.40 ± 0.31 a		2.47 ± 0.11 a	
		15%	46.4 ± 5.5 a		4.10 ± 0.21 a		3.68 ± 0.53 a		2.50 ± 0.11 a	
		30%	50.7 ± 4.2 a		4.27 ± 0.46 a		1.91 ± 0.21 a		2.63 ± 0.30 a	
2014	**ANOVA**	**df**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**p**
	Soil type	3	1.337	>0.05	2.965	>0.05	2.053	>0.05	0.175	>0.05
2015	**Coppiced**	**5%**	78.5 ± 8.9 a		4.04 ± 0.32 b		2.99 ± 0.46 a		3.80 ± 0.29 a	
		10%	62.8 ± 4.4 a		2.99 ± 0.24 a		2.97 ± 0.35 a		3.52 ± 0.10 a	
		15%	57.4 ± 6.0 a		2.87 ± 0.15 a		3.12 ± 0.83 a		3.86 ± 0.23 a	
		30%	65.0 ± 4.6 a		3.76 ± 0.33 ab		2.62 ± 0.22 a		3.25 ± 0.15 a	
2015	**ANOVA**	**df**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**p**
	Soil type	3	2.116	>0.05	5.324	**<0.05**	0.107	>0.05	1.493	>0.05

5.4.3 Nutrient balance

Total Nitrogen and TP concentrations in the soil followed a clear trend at the start of the trial (Figure 5.8). Nutrient concentrations were highest in the 30% clay soil, followed by 5% and then 15% clay soil. The 10% clay soil type showed the lowest levels of TN and TP. At the end of the trial the same trend was found. In general, the TN and TP concentrations increased marginally, but no significant differences between soil nutrient concentrations at the start and end of the trial were found, with the exception of coppiced trees growing in 15% clay, which showed a significantly higher soil TP concentration at the end of the trial than at the beginning, although the actual difference was small.

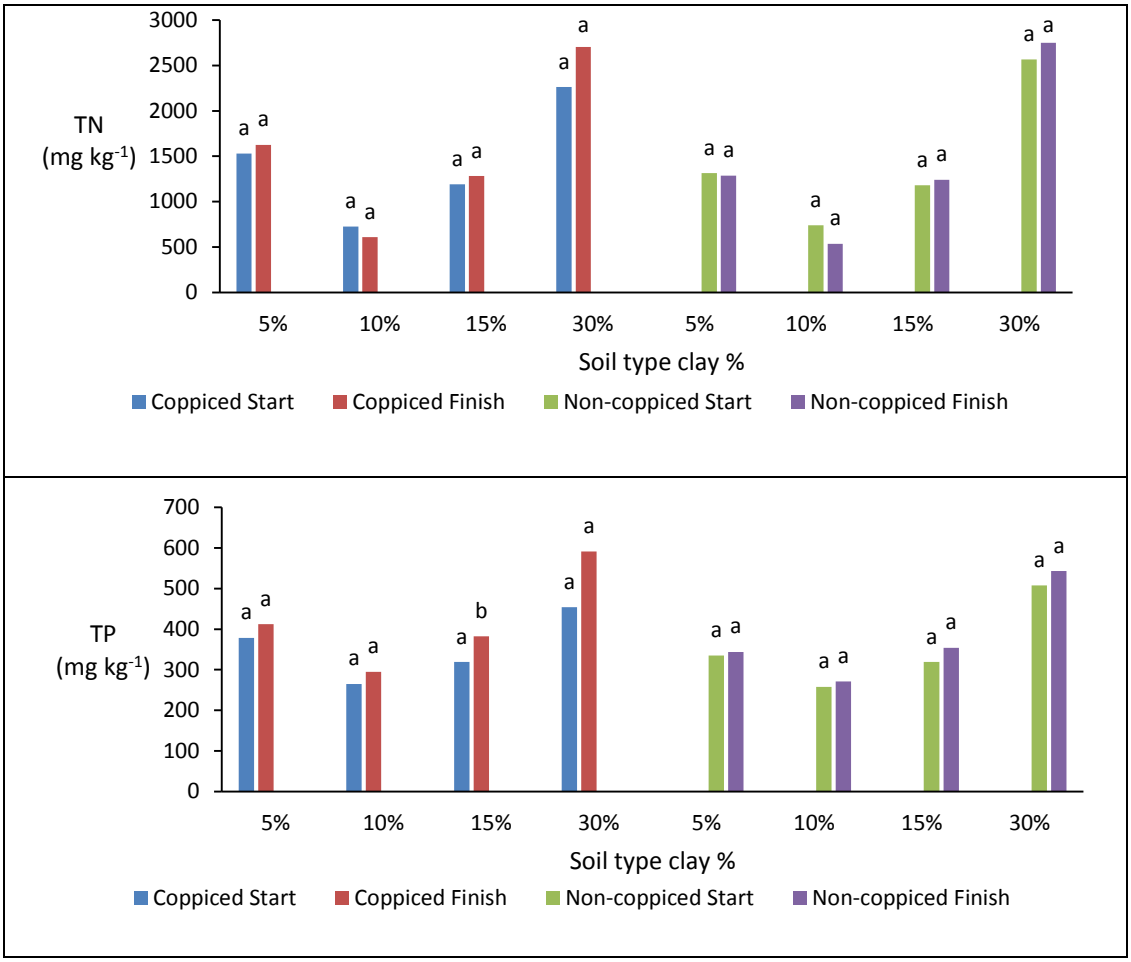
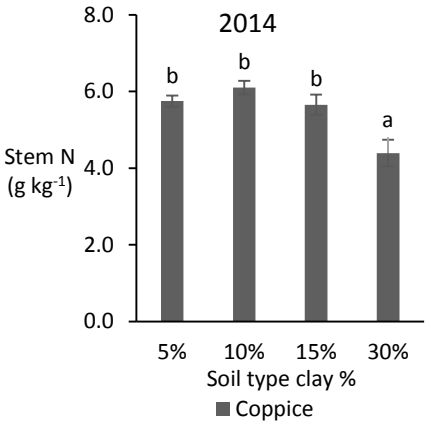
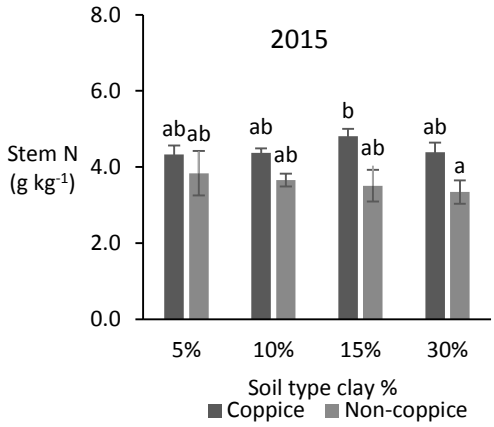


Figure 5.8 Median soil TN (above) and TP (below) at the start and end of the trial. Samples within groups (start and finish) which share a common letter are not significantly different using the Wilcoxon Paired Sample test ($p > 0.05$).

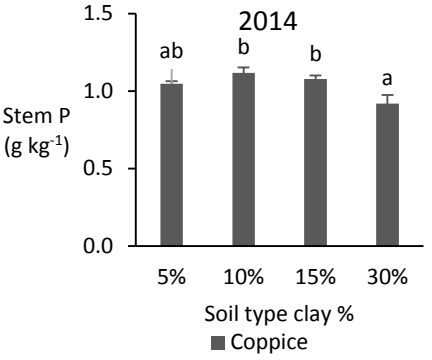
In 2014, stem N concentration of the coppiced trees was significantly lower in the trees grown in 30% clay than in the trees grown in the other three soil types (Figure 5.9). Stem P concentration was lowest in the trees grown in the 30% clay, but was significantly lower than only trees growing in 10% clay and 15% clay. At the end of the trial in 2015, coppiced trees showed significantly higher stem N and stem P concentrations than did non-coppiced trees. Soil type had no significant effect on stem N or stem P, and there was no significant [soil type x management] interaction on either stem N or stem P.

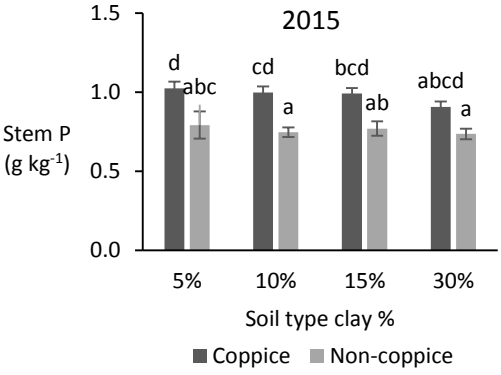




ANOVA	df	F	p
Soil type	3	8.295	**<0.01**
Management	-	-	-
Soil x management	-	-	-

ANOVA	df	F	p
Soil type	3	0.261	>0.05
Management	1	15.110	**<0.001**
Soil x management	3	0.575	>0.05





ANOVA	df	F	p
Soil type	3	6.01	**<0.01**
Management	-	-	-
Soil x management	-	-	-

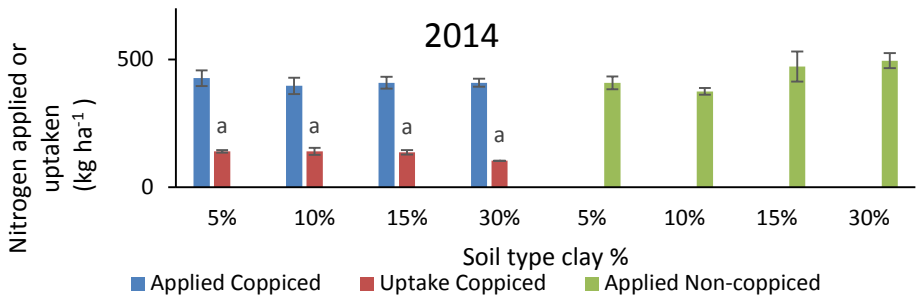
ANOVA	df	F	p
Soil type	3	1.01	>0.05
Management	1	39.43	**<0.01**
Soil x management	3	0.24	>0.05

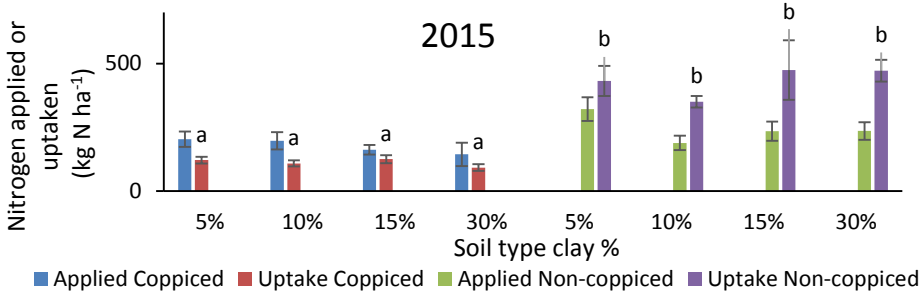
Figure 5.9 Mean ± SE stem nutrient concentration at the end of 2014 (coppiced) and 2015 (coppiced and non-coppiced). Any samples within year which share a common letter are not significantly different using the Tukey test (p > 0.05).

In 2014, no significant differences in stem N removal of the coppiced trees due to soil type were observed, with values varying from 139 kg N ha^{-1} in 10% clay to 103 kg N ha^{-1} in 30% clay (Figure 5.10). Nitrogen application was approximately three times higher than uptake by shoots. Similarly, in 2014 no significant differences in shoot P removal due to soil type were found, with values ranging from 26 kg P ha^{-1} in 15% clay to 22 kg P ha^{-1} in 30% clay. Phosphorus application was approximately

four times higher than uptake by shoots. In 2015, non-coppiced trees removed a significantly higher amount of nitrogen than coppiced trees. There was no significant difference due to soil type and the [soil type x management] interaction was not significant. Harvest of non-coppiced trees led to a significantly greater removal of phosphorus than did the harvest of coppiced trees. There was no significant difference due to soil type, and the [soil type x management] interaction just reached statistical significance ($p = 0.048$). For the coppiced trees, the N and P amounts applied were closer to the amounts removed, while for the non-coppiced trees the amounts of N and P applied were somewhat lower than the amounts of N and P removed through harvesting, though the amount of N and P removed in 2015 reflected a number of years' growth.

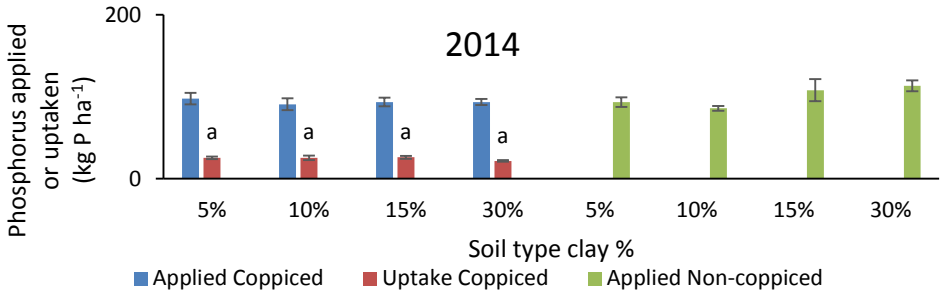
Soil water TN and TP concentrations followed a similar trend to soil TN and TP concentration (Table 5.5). In general, soil nutrient concentrations were lowest in the 10% clay soil type. There were no significant differences in soil water TN or TP concentrations at either time point. For TN, concentrations in the soil water at the summer time point were lower than concentrations during the winter time point. For TP, the concentrations were similar at both time points.

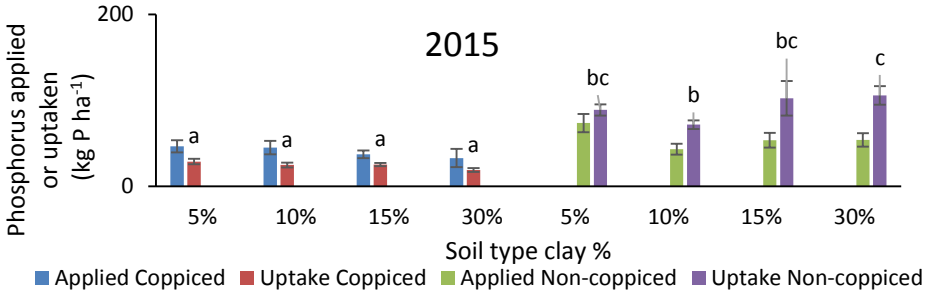




ANOVA	df	F	P
Soil	3	2.645	>0.05
Management	-	-	-
Soil x management	-	-	-

ANOVA	df	F	P
Soil	3	1.109	>0.05
Management	1	177.430	**<0.01**
Soil x management	3	1.154	>0.05





ANOVA	df	F	P
Soil	3	0.733	>0.05
Management	-	-	-
Soil x management	-	-	-

ANOVA	df	F	P
Soil	3	2.036	>0.05
Management	1	163.102	**<0.01**
Soil x management	3	2.976	**0.048**

Figure 5.10 Mean ± SE N and P applied and taken up through harvest in 2014 and 2015. For N and P uptake, any samples within a figure which share a common letter are not significantly different using the Tukey test (p > 0.05).

Table 5.5 Mean ± SE TN and TP concentrations in the soil water at two time points during the trial. Samples within time point which share common letters are not significantly different using the Tukey test (p > 0.05)

Management type	Soil type	TN (mg L^{-1})		TP (mg L^{-1})	
		Winter 2014/15	Summer 2015	Winter 2014/15	Summer 2015
Coppice	**5%**	3.4 ± 0.5 a	2.5 ± 0.6 a	0.16 ± 0.02 a	0.21 ± 0.04 a
	10%	3.3 ± 0.3 a	1.7 ± 0.6 a	0.16 ± 0.03 a	0.18 ± 0.04 a
	15%	3.6 ± 0.3 a	2.2 ± 0.2 a	0.17 ± 0.01 a	0.18 ± 0.02 a
	30%	4.5 ± 0.5 a	2.5 ± 0.3 a	0.24 ± 0.04 a	0.18 ± 0.01 a
Non-coppice	**5%**	4.3 ± 0.3 a	2.0 ± 0.2 a	0.21 ± 0.02 a	0.27 ± 0.07 a
	10%	3.5 ± 0.3 a	1.9 ± 0.2 a	0.22 ± 0.03 a	0.19 ± 0.01 a
	15%	3.4 ± 0.1 a	2.3 ± 0.1 a	0.21 ± 0.02 a	0.21 ± 0.07 a
	30%	3.8 ± 0.3 a	2.5 ± 0.4 a	0.25 ± 0.03 a	0.26 ± 0.06 a

ANOVA	**df**	**F**	**P**	**F**	**P**	**F**	**P**	**F**	**p**
Soil type	3	2.36	>0.05	0.68	>0.05	1.27	>0.05	0.68	>0.05
Management	1	0.09	>0.05	1.75	>0.05	0.09	>0.05	1.75	>0.05
Soil x management	3	2.27	>0.05	0.12	>0.05	0.28	>0.05	0.18	>0.05

5.5 Discussion

Water balance

Seasonal evapotranspiration rates were similar to willow lysimeter evapotranspiration rates found elsewhere in Ireland by Curneen and Gill (2014) and in the UK by Martin and Stephens (2006b). Seasonal evapotranspiration rates were also similar to evapotranspiration rates from full-scale ZDWS in Wexford (Curneen and Gill, 2015). Daily evapotranspiration rates were in agreement with rates reported by Guidi (2008) in Italy, but lower than the peak values of 14-16 mm day^{-1} found by Pistocchi *et al.* (2009), also in Italy. The lower evapotranspiration during the summer of 2015 was likely to be due to slightly lower temperatures in the second year in addition to the increased rainfall and reduced sunlight.

Evapotranspiration rates from the non-coppiced trees were significantly higher than evapotranspiration rates from the coppiced trees during both of the seasons monitored, but the size of the difference varied with both soil type and year. In

2014, trees growing in 15% and 30% clay showed the largest difference in evapotranspiration due to management type. In contrast, in 2015, trees growing in 5% clay showed the largest difference in evapotranspiration between coppiced and non-coppiced trees. Pooled across soil type, in 2014 non-coppiced trees showed an additional 114 mm evapotranspiration on average, which rose to 190 mm in 2015. This increased evapotranspiration of the non-coppiced trees was likely due to their larger size, as biomass is known to be strongly correlated with evapotranspiration (Martin and Stephens, 2006b, 2006a; Białowiec *et al*., 2007). This suggests that the presence of an annual coppicing cycle in a polytunnel-covered system will lower the maximum evapotranspiration that could be attained due to the smaller size of the trees in the polytunnel covered system in comparison to the traditional ZDWS.

However, maximising evapotranspiration is not of utmost importance to a covered ZDWS, but rather maximising wastewater application to the system. In 2014, an unusually dry Irish summer, rainfall was very low (292 mm). During this year, an average of 771 mm of wastewater was applied to coppiced trees, and an average of 806 mm applied to non-coppiced trees. In 2015, an unusually wet summer, the amount of rainfall that fell was much higher (519 mm). During 2015 an average of 336 mm of wastewater was applied to the coppiced trees and 412 mm of wastewater applied to the non-coppiced trees, which was much lower than the previous year. This demonstrates the value of the covered system in preventing the input of rainfall (which is much larger than the loss of evapotranspiration between non-coppiced and coppiced trees), and thereby allowing a greater amount of wastewater to be applied to the same area than would occur in an open system.

A range of soil clay percentages was used here, including a percentage (30%) higher than that normally used in ZDWS which have used soil types of up to 20% clay in Denmark (Ministry of Environment and Energy, 2001). This gave a good range across the predicted clay levels that would be used in ZDWS, as a low permeability site in Ireland will typically have at least 13% clay (Swartz *et al.*, 2003). In both years, non-coppiced trees growing in 10% clay showed the lowest seasonal evapotranspiration. Soil type did not have a significant effect on evapotranspiration at the main effect level in this current study. This is similar to results recorded by Stephens *et al.* (2001) who found that, in the UK, across five soil types (sand, sandy loam, sandy clay loam, silty loam and clay), annual willow evapotranspiration did not differ significantly and varied between 430 and 480 mm.

However, within the non-coppiced trees, those growing in 10% clay showed a significantly lower evapotranspiration rate compared to non-coppiced trees grown in 5% clay. For this particular willow clone, evapotranspiration rates for the non-coppiced trees were lowest in the 10% clay soil, indicating that increased land area would need to be given to the system if the soil used in the ZDWS was close to 10% clay. The low levels of evapotranspiration of the non-coppiced trees in 10% clay could have been due to the lower levels of nutrients (both nitrogen and phosphorus) found in this soil, leading to lower growth (as reflected in the low biomass results) and, hence, lower evapotranspiration. The low nitrogen and phosphorus levels in the 10% clay may have been due to the initial mixing of the soil, where a nutrient-poor batch of soil may accidentally have been used and then mixed with sand, which led to the creation of a particularly nutrient-poor mixture in

the 10% clay lysimeters. For the coppiced trees, the general trend was for evapotranspiration to decrease slightly with increasing clay percentage. High clay soils can tightly bind water molecules, making them less available for soil evaporation and plant transpiration, which may explain this slight decrease (Bellingham, 2013).

The k_c values during the growing season followed a similar trend to those recorded by Persson (1995), Pistocchi *et al.* (2009) and Curneen and Gill (2015), whereby high values were recorded during late summer and autumn. Seasonal values were similar to the values recorded by Guidi (2008) during the first growing season (1.6) and the second growing season (3.0), and to the annual k_c value of 2.5 advised in the Danish ZDWS guidelines (Ministry of Environment and Energy, 2003). The values recorded here are in agreement with the annual value of 2.2 for ZDWS design in Ireland as advised by Curneen and Gill (2015).

Willow growth

As expected, the non-coppiced trees had a much higher biomass at the end of 2015 in comparison to the coppiced trees, as a result of the longer growing cycle. This increased size of the trees led to the higher evapotranspiration from the non-coppiced trees, as willow evapotranspiration is known to be strongly correlated to tree biomass (Curneen and Gill, 2014). Annual yield from the coppiced trees in 2014 was approximately 23-24 odt ha^{-1} yr^{-1} in 2014 and 21-28 odt ha^{-1} yr^{-1} in 2015, which is higher than willow yields of 7-12 odt ha^{-1} yr^{-1} reported elsewhere in Ireland (van den Broek *et al.*, 1997; Styles *et al.*, 2008; Wickham *et al.*, 2010; Dawson, 2011). For the non-coppiced trees, yield at harvest was very high (96 – 144 odt ha^{-1}) which is

reflective of the long cycle in this study. Annual average biomass increase for the non-coppiced trees over the time period was low, however, averaging 10.7 – 16 odt ha^{-1} yr^{-1} over the time period. Cycles of three years are more common, with expected yields of 21-36 odt ha^{-1} expected after a three-year cycle in Ireland (Dawson, 2011). The biomass of the coppiced trees in both years was similar, indicating that annual harvesting did not severely impact growth. Frequent harvesting can lead to a reduction in root reserves, and hence less re-growth (Ceulemans *et al.*, 1996). This was not recorded here, indicating that a short duration of annual coppicing does not appear to affect regrowth. This is similar to results published by Kopp *et al* (2001) in which willow clones that are well adapted to annual coppicing can maintain productivity when coppiced annually. For the non-coppiced trees, there was a significant effect of soil type on biomass. Trees growing in 30% clay had a significantly higher biomass than trees growing in 10% clay, which could be due to the higher levels of nutrients found in the 30% clay soil compared to the 10% clay soil.

Final height of the non-coppiced trees is less than would be expected for a harvest cycle of this length, where growth in Ireland can be 6-8 m after three years (Dawson, 2011). However, it is likely that the trees in this study were limited by the size of the containers and the volume of soil available for growth (approximately 0.17 m^3). Similar to the results found by Herve and Ceulemans (1996) using poplar, SVI was significantly higher in the non-coppiced trees compared to the coppiced trees. There was no clear relationship between the clay gradient and either SVI or

SBA, indicating that, in general, the range of soil types used here are suitable for willow growth.

Maximum LAI recorded here was similar to the maximum LAI recorded by Martin and Stephens (2006b) in the UK for individual willow lysimeters. LAI is known to be related to evapotranspiration (Persson, 1995) and, in both years, trees growing in 30% clay had both the lowest LAI and evapotranspiration of the coppiced trees, indicating lower radiation interception and, hence, lower evapotranspiration. The WUE of the coppiced trees (2.4 – 3.9 g dm kg^{-1} water) was lower than WUE values reported elsewhere for willow (4 - 8 g dm kg^{-1} water)(Linderson *et al.*, 2007; Guidi and Labrecque, 2010). This may have been due to lower soil nutrient levels in the soil used in this study, as low soil nutrient levels are known to lower WUE in willow (Martin and Stephens, 2006b). However, in the context of ZDWS, willows with a lower WUE are desirable as the objective is to remove wastewater, rather than maximise production (Christersson, 2006; Martin and Stephens, 2006b).

Frequent (annual to triennial) coppicing of willow plantations has been shown to lead to survival rates of 73-78% after five years (Kopp *et al.*, 1997). However, in the current study, there was no plant mortality in any of the treatments, suggesting that this particular *S. viminalis* clone is particularly well-adapted to annual coppicing.

Nutrient balance

In general, the N and P concentrations in the soil increased marginally over the course of the trial, but these increases were not significant, with the exception of the P for 15% clay. This agrees with findings reported elsewhere where no

significant differences over time in N and P concentrations beneath willow stands were found (Adegbidi *et al.*, 2003; Quaye and Volk, 2013), but contrasts with the findings of Guidi and Labrecque (2010) who found that soil concentrations of both N and P decreased in a one-year willow pot trial. While the amounts of N and P applied to both coppiced and non-coppiced trees in 2014 were above the recommended range for willow SRC in Ireland (Dawson, 2011), as the overall soil N and P concentrations did not markedly increase, this suggests that the levels of N and P applied over the course of the trial closely matched the nutritional demands of this crop.

Stem N and P concentrations in 2015 were higher in the coppiced trees, which was to be expected, as shorter coppice cycles lead to higher nutrient concentrations in the younger stems (Ericsson, 1994; Adegbidi *et al.*, 2001). For the coppiced trees in 2014, soil type did lead to significant differences in both N and P concentration, with willows growing in 30% clay having significantly lower stem N than trees grown in the three other soil types. This is in contrast to the findings by Labrecque and Teodorescu (2003) who found that willows growing on clay soil had a higher concentration of N and P than willows growing on sandy soil.

For the coppiced trees, no differences in stem N and P content (uptake) due to soil type were found in either 2014 or 2015, with uptake values similar to those reported by Gregersen and Brix (2001) and Curneen and Gill (2014) for willows used in ZDWS. Non-coppiced trees removed significantly larger amounts of nutrients than the coppiced trees in 2015. This was expected due to the longer life cycle and higher biomass of the non-coppiced trees. For the non-coppiced trees, there was a

significant difference in phosphorus uptake. Non-coppiced trees growing in 30% clay had a significantly higher stem P content than did trees growing in 10% clay. As stem P concentrations of these two treatments were not significantly different, this difference was due to differences in productivity, with the greater biomass of the trees growing in 30% clay leading to higher removal rates. This finding is similar to that from other studies on nutrient removal by willow, with differences in yield, rather than concentration, leading to differences in nutrient removal (Aronsson and Bergström, 2001; Rytter, 2001; Dimitriou and Aronsson, 2004).

In the drier summer of 2014, the amounts of N and P added to the coppiced trees were much larger than the amounts removed. By comparison, in the wetter summer 2015, the amounts applied and removed were more similar. This was due to the higher levels of rainfall in 2015, meaning that a lower volume of wastewater could be applied to meet the evapotranspiration demand of the willows in 2015.

Considerable nutrient attenuation took place in the lysimeters, with nutrient concentrations in the soil water reduced by approximately 90%, which is very close to the 90% N reduction and 85% P reduction found by Guidi Nissim *et al.* (2015) for wastewater irrigated willows. Soil water concentrations were similar to the concentrations found by Curneen and Gill (2012) in ZDWS in Wexford, and by Sugiura *et al.* (2008) for wastewater irrigated willow SRC in the UK, suggesting that the lysimeters used in this study are quite similar to the conditions which occur in full-size field systems. Nutrient concentrations in the soil water followed the same trend as was found for the soil N and P. Concentrations were lowest in the 10% clay and highest in the 30% clay. For TN, concentrations were higher during the winter

months than during the summer months. This may be due to lower denitrification during the winter months, as denitrification is known to be temperature dependent and generally lower during the autumn and winter (Lee *et al.*, 2009).

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Chapter 6 The use of mycorrhizal fungi to improve ZDWS performance

6.1 Abstract

The use of mycorrhizal fungi to improve the performance of ZDWS was examined in three phases. In phase 1, rooted *Salix viminalis* cuttings in non-sterile soil were inoculated with commercial mycorrhizal fungal inoculum (DIEHARD^{TM} Transplant) under three different types of wastewater: primary wastewater, tertiary wastewater and rainwater. The height, diameter and shoot biomass of inoculated trees were all significantly greater than in non-inoculated trees, with the biggest difference between inoculated and non-inoculated trees occurring for trees that received rainwater. For most tree parameters, the general growth trend was in the order primary wastewater > tertiary wastewater > rainwater. Similar levels of mycorrhizal colonisation were found under each wastewater treatment and in inoculated and non-inoculated trees. Root inoculum was then collected from phase 1 DIEHARD inoculated trees that received primary wastewater or rainwater ("willow-selected DIEHARD") and non-inoculated trees that received primary wastewater or rainwater ("willow-selected resident mycorrhizal fungi"). In phase 2, the effects of willow-selected DIEHARD and willow-selected resident mycorrhizal fungal inocula on willow growth and mycorrhizal fungal colonisation were compared with those of basic DIEHARD. An ergosterol assay was first conducted to measure and standardise the amounts of fungal material in treatments. At the end of phase 2, trees inoculated with willow-selected DIEHARD had a significantly larger shoot biomass, total leaf area and total leaf weight than trees inoculated with basic DIEHARD. Final height and stem diameter were also larger in trees inoculated with willow-selected DIEHARD compared to basic DIEHARD, but not significantly so. These results show the potential application of willow-specific mycorrhizal fungal

inoculum for use in ZDWS to increase tree growth and evapotranspiration. While differences in mycorrhizal colonisation did occur in phase 2, there were no clear trends between levels of mycorrhizal colonisation and tree responses, indicating the importance of the quality, rather than the quantity, of the mycorrhizal colonisation for tree growth response. In phase 3, using real-time qPCR to identify and quantify individual AMF and ECM, the willow-selected DIEHARD inoculum showed higher proportions of the disturbance-tolerant species *Glomus mosseae*, *G. intraradices* compared to those in basic DIEHARD.

6.2 Introduction

Mycorrhizal fungi form symbiotic associations with approximately 80% of all plant species (Smith and Read, 2008). The *Salix* spp. are dual-mycorrhizal, and form associations with both AMF and ECM (Trowbridge and Jumpponen, 2004; Parádi and Baar, 2006). The presence of mycorrhizal fungi is known to increase nitrogen and phosphorus uptake by plants through enlarging the nutrient interception zone (Fillion *et al.*, 2011; Cavagnaro *et al.*, 2015). ECM have a stronger influence on N nutrition, while AMF have a stronger influence on P nutrition (Smith *et al.*, 1994). Inoculation with AMF or ECM, as well as co-inoculation with both types has been shown to lead to superior willow growth (Wijesinghe, 2012). The presence of AMF has been shown to increase the biomass production of *S. miyabeana* Seemen. and *S. viminalis* L. (Fillion *et al.*, 2011) as well as *Salix x dasyclados* Wimm. (Baum *et al.*, 2006). Inoculation with ten ECM species led to an increase in *Salix repens* L. shoot dry weight compared to the control after 12 weeks (van der Heijden and Kuyper, 2003).

Previous studies have shown a strong positive correlation between willow yield and evapotranspiration rate (Martin and Stephens, 2006a, 2006b; Białowiec *et al*., 2007a; Guidi, 2008; Curneen and Gill, 2014). Hence, increasing willow yield through effective mycorrhizal fungal inoculation could aid in increasing evapotranspiration in ZDWS. In addition, colonisation by AMF can result in an increase in transpiration or stomatal conductance (Allen *et al.*, 1981; Augé, 2001, 2004; Augé *et al.*, 2004). Where the objective (as in a traditional ZDWS) is to increase evapotranspiration

rate, the use of mycorrhizal fungi for improving the effectiveness of ZDWS may be beneficial.

Depending on the land use history e.g. phosphorus fertilisation and cultivation, of the ZDWS site, levels of mycorrhizal fungi in the soil may be low. In disturbed soils, as would be the case in the soil of a ZDWS, mycorrhizal infectivity can be reduced due to the destruction of mycorrhizal networks (Jasper *et al*., 1991; Brito *et al*., 2008). Low levels of mycorrhizal fungi within the soil is a leading cause of poor plant establishment and growth in forestry sites (Ortega *et al.*, 2004). Exploration of the use of mycorrhizal fungal inoculation in ZDWS is therefore warranted.

6.1.1 Willow-selected inoculum

The effect of mycorrhizal inoculation on willow aboveground biomass production varies with type of mycorrhizal inoculant and soil type (Baum *et al.*, 2009; Wijesinghe, 2012). The host plant can also influence the mycorrhizal associations and different mycorrhizal fungi are characteristic for willows grown in natural/naturalised sites and SRC (Hrynkiewicz *et al.*, 2012). Furthermore, mycorrhizal associations are known to be specific to particular willow clones with regard to mycorrhizal colonisation level and host plant response (Baum and Makeschin, 2000; Hrynkiewicz *et al.*, 2012; Fransson *et al.*, 2013).

The effect of mycorrhizal inoculation on host biomass production of four different willow SRC varieties during the first six to twelve months has been shown to vary between positive, neutral and negative, depending on the specific host-mycorrhiza combination (Baum *et al.*, 2009). Willows inoculated with the ECM *Paxillus involutus* (Batsch) Fr., *Tricholoma cingulatum* (Almfelt) Jacobasch or *Hebeloma*

fastibile (Pers.) P. Kumm. performed better than non-inoculated controls, while willows inoculated with the ECM *Laccaria laccata* (Scop.) Cooke had a lower stem biomass production than control plants after six months (Baum *et al.*, 2009). Root lengths of *S. viminalis* cuttings inoculated with the ECM *Laccaria proxima* (Boud.) Pat. were twice as long as those inoculated with the ECM *Thelephora terrestris* (Ehrh.) Fr. after twelve weeks (Jones *et al.*, 1990). In a study of 28 poplar clones, different levels of both ECM and AMF colonisation were recorded, indicating different host receptivity to colonisation (Khasa *et al.*, 2002). For *Salix repens*, the AMF *Glomus mosseae* Gerd and Trappe was more beneficial for shoot and root growth in the short term after establishment (12 weeks), but in the longer term (30 weeks) the ECM *Hebeloma leucosarx* P.D. Orton can be more beneficial (van der Heijden, 2001).

It is a matter of debate whether specific fungal inocula or broad-spectrum generic inocula are better suited to the commercial mycorrhizal inoculum market (Smith and Read, 2008). Molina *et al.* (1992) noted that the use of inocula which are specialised for a particular host or habitat is potentially more beneficial than developing inocula for broad-spectrum application. This is important considering that Rooney *et al.* (2011) have shown that AMF can reduce *Populus euramericana* cv. Ghoy stem dry weight in comparison to non-inoculated controls, indicating that care must be taken when choosing the mycorrhizal fungi species to use as inoculum.

While mycorrhizal colonisation levels can vary between willow species (Püttsepp *et al.*, 2004), Baum *et al.* (2009) observed that the identity of the fungal species

forming the association, rather than the degree of colonisation, is particularly important for host plant response. Furthermore, different ecotypes of the same mycorrhizal fungal species can vary in their effects on the host plant (Baum *et al.*, 2006). Differential responses of host plant species to individual isolates and species of mycorrhizal fungi (Streitwolf-Engel *et al*., 1997; Kennedy *et al*., 2007) have been reported, leading to variations in plant productivity depending on the diversity and identity of the mycorrhizal fungi (van der Heijden *et al*., 1998; Klironomos *et al*., 2000). Ortega *et al.* (2004) stated that, for afforestation to be successful, it is essential that nurseries produce mycorrhized tree species that are ecologically adapted to the tree and the planting site. This suggests that certain species that are adapted to both clone and site factors, e.g., clay soil, primary wastewater application and short coppice cycle, should be trialled as willow-selected inoculum to best enhance willow establishment and growth in ZDWS.

Although spore morphology characteristics have been used to identify AMF in soil, this approach has limited applicability to the situation in roots (Clapp *et al*., 1995; Jansa *et al*., 2003). Molecular approaches, reviewed by Krüger *et al.* (2009), have taken over, with real-time quantitative polymerase chain reaction (qPCR) becoming established as the tool-of-choice to quantify abundance of different mycorrhizal fungi in both roots and soil (e.g. Thonar *et al.* (2012)). In this study, qPCR markers (i.e. DNA primers with associated hydrolysis probes) targeting taxon-specific motifs in the nuclear large rRNA subunit gene (Thonar *et al*., 2012) will be used to identify and quantify individual mycorrhizal fungi present in the original DIEHARD inoculum and to compare them to the proportions present in the inoculated willow roots, to

determine whether there is evidence for selection of mycorrhizal species by colonisation of willow roots with a mixed-species inoculum.

6.1.2 Objectives

The objectives of the research described in this chapter are:

(a) to investigate the effect of commercial mixed-species mycorrhizal fungal inoculum on the growth of *Salix viminalis* trees (phase 1)

(b) to compare the performance of a willow-selected mycorrhizal fungal inoculum with a commercial mixed-species mycorrhizal fungal inoculum (phase 2)

(c) to compare the relative proportions of AMF and ECM taxa in willow-selected inoculum and a commercial mixed-species mycorrhizal fungal inoculum (phase 3)

6.3 Materials and methods

There were three phases in this trial. Phase 1 assessed the response of *S. viminalis* cuttings to a commercial mixed-species (AMF and ECM) mycorrhizal fungal inoculum (DIEHARD^{TM} Transplant, Horticultural Alliance, Sarasota, Florida, USA) in the presence of three different wastewater types and two different irrigation rates. Phase 2 examined the potential for developing a willow-selected mycorrhizal fungal inoculum from a commercial mixed-species inoculum by using roots from DIEHARD-inoculated trees (phase 1) as inoculum. Both trials were carried out in the School of BEES, UCC. In phase 3, qPCR was used to identify and quantify the mycorrhizal species present in the inoculum used in phase 3. A schematic of these methods is shown in Figure 6.1**.**

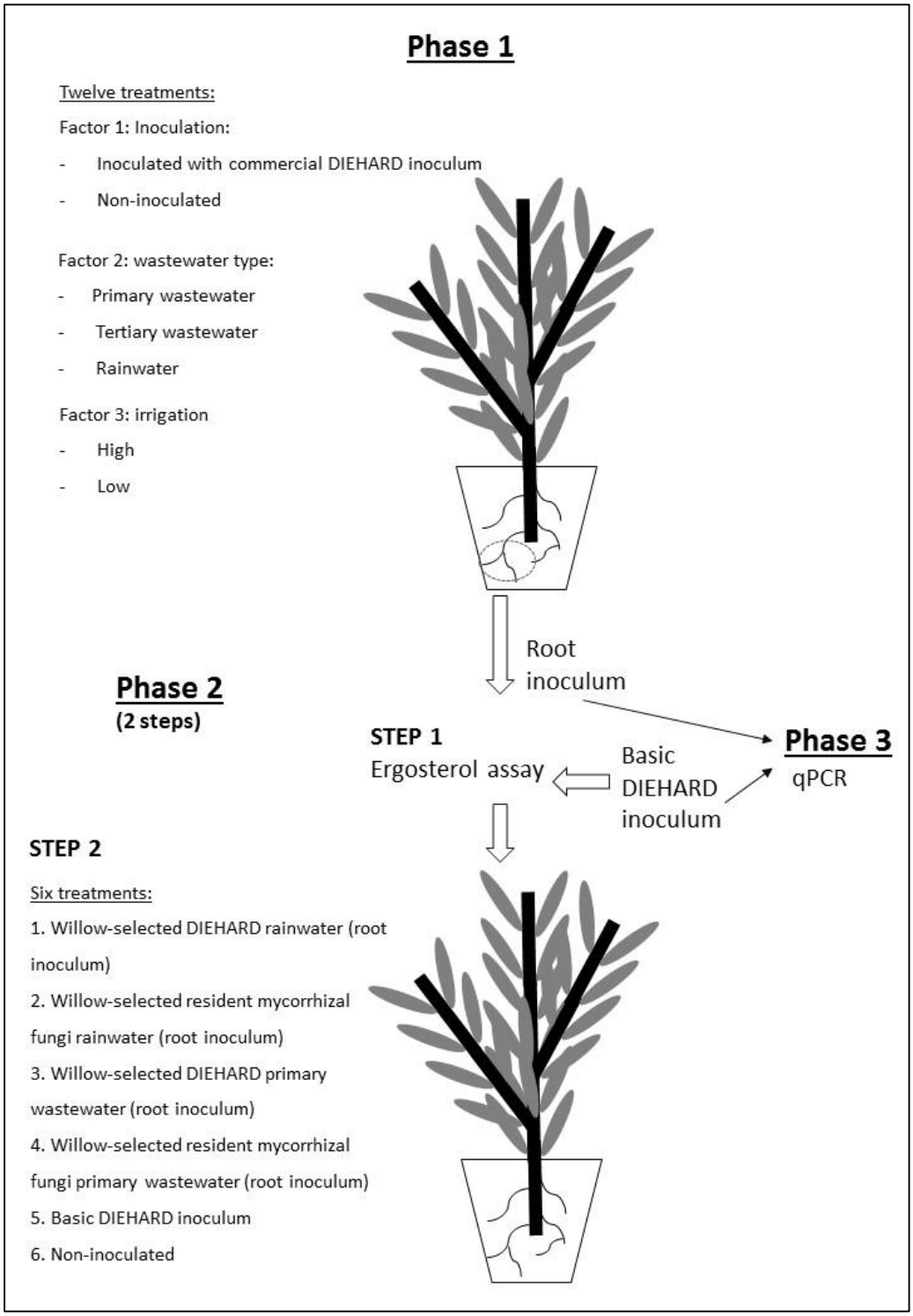


Figure 6.1 Schematic of phases 1-3.

6.3.2 Phase 1 - response to commercial mycorrhizal fungal inoculum

6.3.2.1 Experimental setup

Soil with a high percentage of silt and clay from a poorly drained site was used, as this would be representative of the soil type used in a ZDWS. The soil was taken from a formerly agricultural site in Tyone Mills, Co. Tipperary (grid reference: R 875 775) and was supplied by Landtech Soils Ltd. Soil pH, electrical conductivity, organic matter content and texture analysis were carried out following the methods described in section 3.3.1 (Table 6.1).

Table 6.1 Characteristics of the soil used in phases 1 and 2.

Parameter	Value
pH	7.5
Electrical conductivity$_{1:5}$ (µS cm^{-1})	141.9
Organic matter content (% LOI)	3.47
Sand (%)	41.3
Silt (%)	46.5
Clay (%)	12.2

S. viminalis rods, taken from the Cloyne field site, were used for the propagation material. Rods were cut to 25 cm in length and a minimum diameter of 8 mm. Cuttings were soaked overnight in distilled water and surface sterilised with 6% hydrogen peroxide for one minute and rinsed for twenty minutes in three changes of distilled water (van der Heijden and Kuyper, 2003) before planting in the rooting medium. A 50/50 mixture of autoclaved vermiculite and perlite was used as rooting medium.

After four weeks, cuttings with a root length of at least five centimetres were selected. Pots (10 L volume) were sterilised with alcohol. A sterilised geotextile disc

was placed at the bottom of each pot to retain the soil. Large stones were removed from the soil by hand. There were three factors (Figure 6.1): inoculation with DIEHARD Transplant^{TM} (inoculated and non-inoculated), wastewater type (synthetic primary wastewater, synthetic tertiary wastewater and rainwater), and irrigation rate (high: 9 L per week, low: 3 L per week). Each of the twelve treatments was replicated ten times, with one tree per pot.

6.3.2.2 Wastewater type

Synthetic primary wastewater (section 4.3.2) and synthetic tertiary wastewater (section 4.3.2) were used for irrigation. Rainwater was collected from the adjacent glasshouse roof in the School of BEES.

6.3.2.3 Inoculation

At the time of planting, cuttings undergoing the inoculation treatment were each inoculated with 28 g of DIEHARD Transplant inoculum as per the supplier's guidelines. DIEHARD is a broad-spectrum commercial inoculum containing AMF, ECM and plant growth promoters. The AMF and ECM species are shown in Table 6.2. While DIEHARD is an American product, the AMF and ECM isolates are of European origin. The full list of DIEHARD constituents is contained in Appendix 1. Inoculum was mixed into the soil in the area surrounding the root ball to ensure good contact with the roots. Cuttings were allowed to establish for four weeks. During this period the pots were watered with tap water. The pots were located outdoors during this period. Inoculated pots were located at least 2 m from non-inoculated pots to reduce the risk of contamination (Fillion *et al.*, 2011). Pots were also placed on a grid to prevent the possibility of cross-contamination of inoculum

from any pooling of irrigation water that occurred at the base of the pots. Within the inoculation factor, plants were arranged in a randomised block design. At the end of the four weeks the pots were moved indoors to a glasshouse with natural sunlight supplemented by artificial light (400 W sodium vapour lamps) with a photoperiod of a sixteen-hour day (average temperature 18 °C) and an eight-hour night (average temperature 10 °C). Pots remained in the glasshouse for a further nine weeks until the end of the trial. Pots were moved indoors to prevent the trees entering dormancy as the total experimental period was for thirteen weeks (September to December).

Table 6.2 DIEHARD^{TM} Transplant AMF and ECM constituents

AMF
12.5 propagules per cc:
Glomus mosseae
Glomus intraradices
Glomus claroideum
Glomus etunicatum
Glomus clarum
Glomus deserticola
Glomus microaggregatum
ECM
180,400 colony forming units per cc:
Pisolithus tinctorius (155,400)
Rhizopogon occidentalis (25,000)

6.3.2.4 Mycorrhizal colonisation

At the end of phase 1, roots were sampled from five pots from each treatment. Two cores were taken per pot, using a stainless steel soil corer that was sterilised with 70% alcohol between uses. Cores were soaked in water for 1 hour and roots were removed using a forceps and placed in glass vials. The soil was then sieved through 1 mm and 250 µm sieves, with roots removed from the sieve grating. Sampled roots

were stored in 50% alcohol at 4 °C for further analysis. Roots were initially analysed under a dissecting microscope (Brundrett *et al.*, 1996). Mycorrhizal fungi were not visible using this method and further analysis was carried out using a compound microscope. Roots were first rinsed in distilled water, and cleared in 10% potassium hydroxide solution by heating in a water bath at 90 °C for four hours, with one change of potassium hydroxide solution after two hours. Where roots did not clear well, 5% hydrogen peroxide was used to clear darkly-coloured material. Cleared roots were rinsed in distilled water, and stained using a 0.5% (v/v) ink and vinegar solution (Vierheilig *et al.*, 1998) by heating in a water bath at 90 °C for one hour. Roots were destained by rinsing in distilled water with 2-3 drops of vinegar. Destained roots were cut into 2-4 cm long pieces and analysed under a compound microscope at 400x magnification with 150 fields of view taken for each sample (McGonigle *et al.*, 1990).

6.3.2.5 Tree measurements

Tree height was measured weekly from the point of emergence from the cutting to the apex of the leading stem. The diameter at the base of the leading shoot was measured at the end of the trial using digital callipers. Oven dry weight of the shoot biomass (dried at 60°C for one week), the total leaf dry mass and the shoot number were recorded at the end of the trial. Leaf area was recorded by selecting the ten uppermost fully developed leaves. These leaves were photographed and leaf area analysis was carried out using Image J image analysis software (National Institutes of Health, Maryland, USA). Specific leaf area (SLA) (leaf area/leaf dry weight) was calculated following leaf drying in an oven at 60 °C for 48 hours.

6.3.3 Phase 2 – development of a willow-selected mycorrhizal fungal inoculum

There were two steps in phase 2. In step 1 an ergosterol assay was carried out to quantify mycorrhizal fungi presence in different sources of mycorrhizal inoculum. The second step followed from the results of the ergosterol analysis and measured the willow growth response and mycorrhizal colonisation resulting from the different sources of mycorrhizal fungi inoculum.

6.3.3.1 Step 1 - Ergosterol assay of willow roots and basic DIEHARD inoculum

In phase 2, an ergosterol assay was first carried out to measure the amount of fungal material in the willow-selected DIEHARD root inoculum from phase 1 and the amount of fungal material in a basic version of DIEHARD inoculum containing only the mycorrhizal fungi. The objective of the ergosterol assay in phase 2 was to measure and standardise the amount of fungal material in the willow-selected DIEHARD root inoculum from phase 1 with the amount of fungal material in the basic version of DIEHARD. The "basic DIEHARD" inoculum was supplied by Horticultural Alliance and contained only the fungal constituents shown in Table 6.2. It did not contain any of the plant growth promoting constituents in the original DIEHARD Transplant used in phase 1.

Mycorrhizal roots can be used as a source of mycorrhizal inoculum (Biermann and Linderman, 1983; Brundrett *et al.*, 1996). For the willow-selected root inoculum, four treatments from phase 1 were selected:

- willow-selected DIEHARD primary wastewater irrigated

- willow-selected DIEHARD rainwater irrigated

- willow-selected resident mycorrhizal fungi (i.e. non-inoculated) primary wastewater irrigated

- willow-selected resident mycorrhizal fungi (i.e. non-inoculated) rainwater irrigated.

The ergosterol assay was carried out following the method of Mohd As'wad *et al.* (2011). For the root-based samples, roots were sampled from five high- and five low-irrigation pots within each treatment. The ten high- and low-irrigation samples were then paired and combined to give five replicates per treatment. Roots were stored in HPLC grade methanol until processed (Newell *et al.*, 1988). Prior to ergosterol analysis, willow roots were frozen at - 80 °C followed by freeze-drying (Nylund and Wallander, 1992; Olsrud *et al.*, 2004). For the basic DIEHARD treatment, samples of 100 – 1,000 mg powdered inoculum were used.

A sample of 100-200 mg freeze-dried roots or powdered inoculum was placed in a 20 ml polyethylene scintillation vial, along with acid-washed beads (2 g of 212 – 300 µm diameter and 0.5 g of 710-1180 µm diameter)(Sigma, Dublin, Ireland) and 8 ml HPLC-grade methanol. Vials were vortexed twice each for 30 seconds, shaken on a bench top shaker for 90 minutes and then allowed to settle for 15 minutes. A 1.5 ml aliquot was transferred to a microcentrifuge tube and centrifuged at 14,000 x g for 10 minutes. Following centrifugation, 0.9 ml of the supernatant was filtered through a 0.2 µm sterile PTFE filter into a 2 ml pre-slit sample vial (VWR, Dublin, Ireland). Vials were loaded on to a Waters UPLC H-Class Core system (Waters Corporation, Milford, Massachusetts, USA) for analysis. Extracts were eluted with HPLC-grade methanol at a flow rate of 0.92 ml minute^{-1} and the eluate monitored

at 282 nm. The column used was an Acquity UPLC BEH C18 1.7µm, 2.1 x 50 mm column (Waters Corporation, Milford, Massachusetts, USA). The detector was an Acquity UPLC TUV Detector (dual wavelength). Under these conditions the peak retention time was 26 seconds. Pure ergosterol (Sigma, Dublin, Ireland) was dissolved in methanol and used for spiking and to prepare the standard curve. Recovery was estimated from the difference between the ergosterol recovered between the spiked and non-spiked samples, divided by the amount of ergosterol spiked (Gong *et al.*, 2001).

6.3.3.2 Step 2 – willow growth trial of root inoculum and basic DIEHARD

There were six treatments in this step of phase 2 (Figure 6.1); the four root-based inocula, basic DIEHARD and a control treatment of non-inoculated trees.

Willow cuttings were prepared as before (section 6.3.2.1), except that 70% alcohol was used for surface sterilisation instead of hydrogen peroxide. The amount of root inoculum or basic DIEHARD inoculum applied was equalised in terms of ergosterol (Hart and Reader, 2002). Where no root ergosterol was detected, root inoculum treatments were equalised in terms of root weight. For the four root inoculum treatments, willow roots were cut from the phase 1 trees, washed, blotted dry and applied near the root ball of the phase 2 root-based inoculum treatments. For the basic DIEHARD treatment, the powdered inoculum was applied near the root ball. Control cuttings were not inoculated. Trees were located outdoors on a grid (section 6.3.2.1) in a replicated randomised block design, and were irrigated with rainwater collected from the adjacent glasshouse roof in times of dry weather. The trial was run for fourteen weeks (July – October). There were ten replicates per

treatment. Mycorrhizal colonisation and aboveground measurements were made following the methods described in sections 6.3.2.4 and 6.3.2.5. Total tree leaf area was calculated as the product of SLA and total tree leaf dry weight.

6.3.4 Phase 3 – qPCR analysis

The research described in Phase 3 was carried out by Dr. Steffan Hamulka, UFZ, Saale, Germany on plant and fungal material supplied by this author. Phase 3 used real-time qPCR to identify and quantify those AMF and ECM taxa from DIEHARD which had colonised the willow roots, and to identify any changes in the proportions of the mycorrhizal fungi after passage through willow roots, as a first step to identifying any evidence of selection for specific mycorrhizal fungi, and to relate such selection to any observed effects on the inoculated willow trees in phase 2.

Root samples were collected from the willow-selected DIEHARD rainwater irrigated roots and the willow-selected resident mycorrhizal fungi rainwater irrigated roots from phase 1. Roots were then freeze-dried. Fifty mg of freeze-dried roots or basic DIEHARD were used for analysis. The method used for identification and quantification of individual mycorrhizal fungal species in DIEHARD inoculum and willow roots was that of Thonar *et al.* (2012). Taxon-specific primers based on the nuclear large ribosomal subunit (nLSU) gene were designed for each of the target AMF and ECM isolates present in DIEHARD (Table 6.2) using the AlleleID version 4 software (Premier Biosoft International, Palo Alto, California, USA). Care was taken to target conserved sequence motifs, which showed little variability across different

gene variants cloned from the same isolate. These were subsequently tested for cross-reactivity using gDNA from spores of the target isolates.

Cross-specificity of the designed primer pairs was tested with both ordinary PCR coupled with agarose gel electrophoresis and by qPCR (using the LightCycler® FastStart DNA MasterPLUS SYBR Green I kit and LightCycler 2.0 instrument, both from Roche Diagnostics, Rotkreuz, Switzerland). Low-stringency conditions (annealing time 90 s, annealing temperature 48 °C, elongation time 60 s, 40 cycles) were employed for both approaches. One primer pair was selected for each isolate on the basis of showing both strong amplification with the target isolates and a complete absence of amplification with non-target isolates.

The selected primers were further complemented by hydrolysis probes, designed using the Allele ID software version 6. The probes were synthesised and labelled with fluorescein (5') and BHQ-1 quencher (3'), and subsequently purified by PAGE in Microsynth. The resulting sets of specific markers (i.e. primers and hydrolysis probes) for the different taxa are shown in Table 6.3. Different cycling conditions were tested to optimise the qPCR conditions for each set of markers with respect to the amplification efficiency (i.e. deviation from optimal cycling conditions postulating duplication of DNA amount every cycle) and specificity. Optimised cycling conditions were established as follows: initial DNA denaturation and DNA polymerase activation at 95 °C for 15 min, then 45 cycles each with denaturation at 95 °C for 10 s, annealing at temperature optimised for each marker for 30 s and elongation at 72 °C for 1 s. Reaction volume was 9 µL (4.52 µL H_2O + 0.18 µL each

primer (25 µM) + 0.07 µL hydrolysis probe (25 µM) + 1.8 µL Roche Master Mix TaqMan + 2.25 µL template).

Table 6.3 Sequences of primers and hydrolysis probes used for the quantitative real-time PCR quantification of large ribosomal subunit gene copies of the different mycorrhizal fungal taxa.

Target AMF species	Sequences (5′→3′) (primer forward, primer reverse, hydrolysis probe)	Amplicon size (base pairs)
Glomus intraradices Schenck & Smith	TTCGGGTAATCAGCCTTTCG TCAGAGATCAGACAGGTAGCC TTAACCAACCACACGGGCAAGTACA	250
Glomus claroideum Schenck & Smith	GCGAGTGAAGAGGGAAGAG TTGAAAGCGTATCGTAGATGAAC AACAGGACATCATAGAGGGTGACAATCCC	177
Glomus mosseae Gerd & Trappe	GGAAACGATTGAAGTCAGTCATACCAA CGAAAAAGTACACCAAGAGATCCCAAT AGAGTTTCAAAGCCTTCGGATTCGC	122
Glomus clarum Nicolson & Schenck	CTTTGAAAAGAGAGTTAAATAG GTCCATAACCCAACACC TAACCTGCCAAACGAAGAAGTGC	272
Glomus microaggregatum Koske, Gemma &P.D. Olexia	AGAAACGTTTTTTACGTTCCGGGTTG CCAAACAACTCGACTCTTAGAAATCG CCGTGTATACCAACCACTGGAATGTTATT	157
Target ECM species	**Sequences (5′→3′) (primer forward, primer reverse, hydrolysis probe)**	**Amplicon size (base pairs)**
Rhizopogon occidentalis Dexter & C.W. Dodge	ACCTCTGCATGGTTTTGGTA AGACATAGGTACTTTGTCCTTTCC CATGAACTGTGCTAGAGCCTCTCT	185
Pisolithus tinctorius (Pers.) Coker &Couch	CCCTCTACATTGGTTTTGGTA AGACATAGGTACTTTGTCCTTTCC GTGTATCACACCACTTGGAATGTTAT	213

The calibration of the qPCR analyses was carried out in two ways, to allow for the conversion of the qPCR output (i.e. Cq) into (i) nLSU gene copy numbers and (ii) amount of genomic DNA (gDNA) of each of the mycorrhizal fungi. The qPCR

quantification protocol for nLSU copy numbers was established following the method of Thonar *et al.* (2012). Briefly, DNA was extracted from single spores of the target isolates and further used as templates for normal PCR with LR1 (5'-GCATATCAATAAGCGGAGGA-3') and FLR2 (5'-GTCGTTTAAAGCCATTACGTC-3') primers, using the following cycling conditions: initial denaturation at 95 °C for 5 min; 35 cycles: denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min and elongation at 72 °C for 1 min; final elongation at 72 °C for 5 min. The concentration of nLSU copy numbers (NC, copies/L) in each of these PCR products was calculated using the amplicon length, *L* for each isolate, the DNA concentration of the sample (*K,* g/L, determined by UV spectrophotometry) and molecular weight of DNA (660 Da/bp) in the following equation, where N_a is Avogadro's constant (6.022×10^{23}):

$$NC = \frac{K \times N_a}{660 \times L}$$ **Equation 6.1**

Then, the PCR products, harbouring the different sequence variants occurring in each individual isolate, were serially diluted to obtain multiple nLSU copies/µL. These dilution series were run through the qPCR with their respective markers under the optimised cycling conditions for each marker. Based on these analyses, calibration curves were established allowing the conversion of qPCR output into the concentration of nLSU copies of each taxon in a sample. Similarly, calibration curves were established for the conversion of the qPCR output to concentration of gDNA of each particular taxon per sample by using serial dilution of gDNA extracted from the target isolates. This calibration with gDNA was subsequently also carried out for all other isolates for which sufficient amounts of gDNA were available; sufficient

gDNA of *G. deserticola* was not available, so this species was consequently omitted from these studies.

For those isolates for which enough gDNA was available to carry out the calibration, the ratio of detected nLSU gene copies per unit amount of gDNA was calculated. Namely, the calibration curves for nLSU copy numbers were used to calculate Cq (signal strength) values for theoretical 10, 100, 1000 and 10 000 detected nLSU copies per microlitre of template. These four Cq values per isolate were subsequently used to calculate the amount of gDNA per microlitre of template, and hence to calculate the mean ratio of nLSU copies ng^{-1} gDNA.

For estimates of colonisation of the plant roots by individual mycorrhizal fungi, the DNA was extracted from 30 mg of lyophilised inoculum or willow roots using the Qiagen Plant DNeasy kit, following the manufacturer's instructions. Each sample was spiked with an internal DNA standard before extraction to normalise rates of DNA extraction efficiencies between the samples and also to test for the presence of PCR inhibitors. To this end, 2^{10} copies of the linearised plasmid carrying fragment of cassava mosaic virus DNA (GenBank accession number AJ427910) was used, and its recovery after the extraction was quantified by qPCR. The following qPCR marker system was used for the internal standard: forward primer (5¢–3¢): CGAACCTGGACTGTTATGATG, reverse primer: AATAAACAATCCCCTGTATTTCAC, and a hydrolysis probe: fluorescein-5¢-CACCAGGCACCAACAACGACCATT-3¢-BHQ1 quencher. Cycling conditions were as described earlier, with the annealing temperature being 50 ^{o}C. The DNA recovery rates of the internal standard for each

individual DNA sample were used to correct the qPCR results obtained with the taxa-specific markers, as described previously (von Felten *et al*., 2010).

6.3.5 Statistical analysis

All variables were normal or normalised by appropriate data transformation. Growth parameters and colonisation rates for phase 1 trial data were analysed using two-way ANOVA. Tree height data were square root transformed to normalise. Shoot biomass dry weight data and average leaf area data were log transformed. Multiple comparisons were conducted using the Tukey test. SLA, total plant leaf biomass and shoot number data were analysed using non-parametric two-way ANOVA. In these cases, multiple comparisons were carried out using the Kruskal-Wallis multiple comparison test. Chi-square analysis was used to assess differences in survival. Tree growth and colonisation rates for the phase 2 trial were analysed using a one-way ANOVA. For phase 2, shoot number, average leaf area, SLA and mycorrhizal colonisation data were square root transformed to approximate to normality. Total leaf biomass, average leaf dry weight and total tree leaf area data were log transformed. Multiple comparisons on normal (or normalised) variables were conducted using the Tukey test. Data from phase 3 were non-normal and were not normalised, so were analysed using the Kruskal-Wallis test. Descriptive statistics are presented as non-transformed values. Statistical analyses were carried out using SPSSv20.

6.4 Results

6.4.1 Phase 1 - response to commercial mycorrhizal fungal inoculum

6.4.1.1 Mycorrhizal colonisation

Irrigation did not have a significant effect on any of the plant growth responses or colonisation, and the results for high and low irrigation were therefore pooled for all subsequent analyses. Mycorrhizal colonisation was detected in all treatments (inoculated and non-inoculated), indicating that colonisation of the non-inoculated trees arose from the resident mycorrhizal population in the soil (Table 6.4). No significant differences in root mycorrhizal colonisation were found between any of the treatments. There was no significant interaction. The highest average level of mycorrhizal colonisation of 78.3% occurred in the non-inoculated primary wastewater irrigated trees. The lowest average level of mycorrhizal colonisation of 59.9% occurred in the DIEHARD-inoculated primary wastewater irrigated trees.

Table 6.4 Mean ± SE willow root mycorrhizal colonisation at the end of phase 1. Any samples which share a common letter are not significantly different using the Tukey test (p > 0.05).

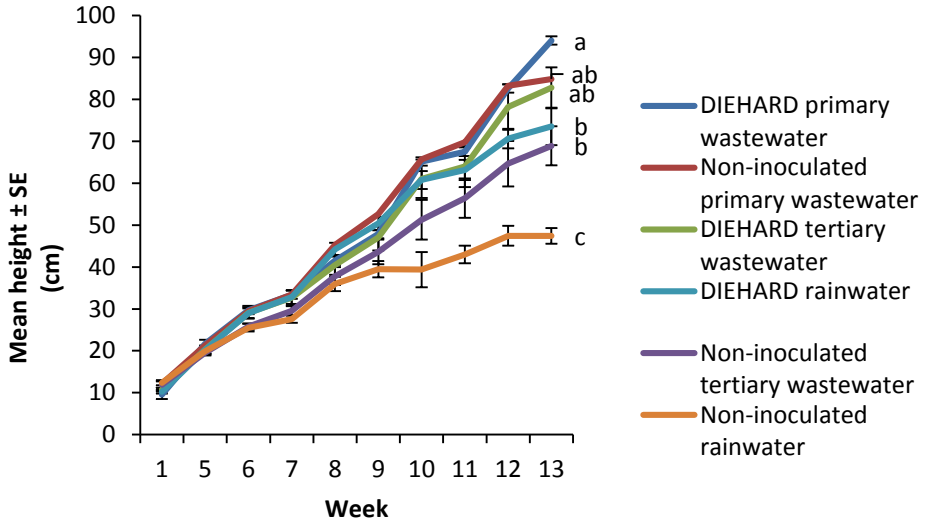
Treatment Inoculation	Wastewater type	Root colonisation (%)
DIEHARD-inoculated	Primary	59.9 ± 6.4 a
	Tertiary	65.3 ± 4.9 a
	Rainwater	64.5 ± 5.6 a
Non-inoculated	Primary	78.3 ± 4.4 a
	Tertiary	62.8 ± 6.8 a
	Rainwater	67.7 ± 4.9 a

ANOVA	df	F	p
Inoculation	1	1.98	>0.05
Wastewater type	2	0.41	>0.05
Inoculation x wastewater type	2	1.88	>0.05

6.4.1.2 Tree growth

Differences in height began to emerge after approximately seven weeks (Figure 6.2). At the end of the trial, DIEHARD-inoculated primary wastewater irrigated trees were the tallest, reaching a final average height of 94 cm. Non-inoculated rainwater irrigated trees were the smallest, with a final average height of 47 cm. Both inoculation and wastewater type had a significant effect on final height. There was also a significant [inoculation x wastewater type] interaction (Figure 6.2). For DIEHARD-inoculated trees, those that received primary wastewater were significantly taller than trees that received rainwater. Trees that received tertiary wastewater were not significantly different in height from trees that received primary wastewater or rainwater. For non-inoculated trees, there was a significant difference in height between all three wastewater types, with trees that received primary wastewater being tallest at the end of the trial, and trees that received rainwater being the shortest. For trees that received rainwater, inoculated trees

were significantly taller than non-inoculated trees. Inoculation did not lead to a significant difference in height for trees receiving primary or tertiary wastewater.

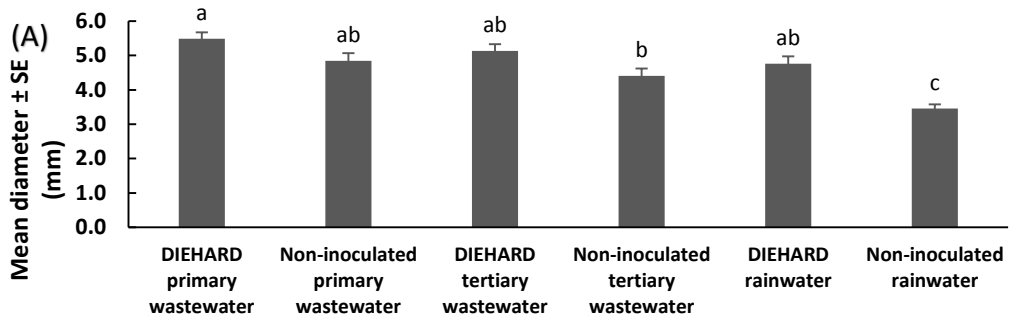


ANOVA	df	F	p
Inoculation	1	23.03	**<0.001**
Wastewater type	2	24.18	**<0.001**
Inoculation x wastewater type	2	3.26	**<0.05**

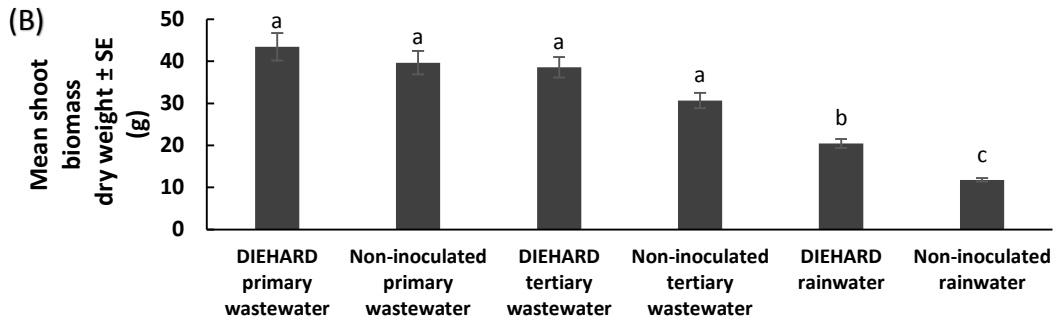
Figure 6.2 Mean ± SE height of leading stem during phase 1. Any week 13 samples which share a common letter are not significantly different using the Tukey test (p > 0.05).

A similar trend was observed for final stem basal diameter, with DIEHARD-inoculated primary wastewater irrigated trees being the largest (5.49 mm) while non-inoculated rainwater irrigated trees were the smallest (3.46 mm) (Figure 6.3). Final stem diameter in inoculated trees was significantly larger than that for stems of non-inoculated trees. Trees that received primary wastewater or tertiary wastewater had significantly larger diameters than trees that received rainwater. There was no significant [inoculation x wastewater type] interaction (Figure 6.3). Both inoculation and wastewater type had a significant effect on final shoot biomass, and there was also a significant [inoculation x wastewater type] interaction (Figure 6.3). Trees that received primary or tertiary wastewater were

significantly heavier than trees that received rainwater. Inoculation had a significant effect for trees that received rainwater, with inoculated trees significantly outperforming non-inoculated trees. There was no significant difference between trees that received primary or tertiary wastewater, irrespective of inoculation.



ANOVA	df	F	p
Inoculation	1	30.43	**<0.001**
Wastewater type	2	14.74	**<0.001**
Inoculation x wastewater type	2	1.65	>0.05



ANOVA	df	F	p
Inoculation	1	20.38	**<0.001**
Wastewater type	2	77.74	**<0.001**
Inoculation x wastewater type	2	3.84	**<0.05**

Figure 6.3 Mean ± SE final diameter (A) and dry weight harvested (B) at the end of the phase 1. Any samples which share a common letter are not significantly different using the Tukey test (p > 0.05).

For both average leaf area and average leaf weight, wastewater type had a significant effect (Table 6.5).

Table 6.5 Final leaf parameters and shoot growth results at end phase 1.

Treatment		Average leaf area (mm^2)	Average leaf dry weight (mg)	SLA ($cm^2\ g^{-1}$)^x	Total tree leaf dry weight (g)^x	No. shoots $plant^{-1x}$
Inoculated	Primary	155.8 ± 11.5 a	14.0 ± 0.9 a	115.7 (106.6-125.5) a	4.8 (4.2-5.6) a	6.0 (4-7) a
	Tertiary	241.5 ± 28.1 b	19.9 ± 1.7 b	117.7 (105.1-122.2) a	4.6 (3.9-5.3) a	5.5 (4-7) a
	Rainwater	230.6 ± 22.5 ab	19.2 ± 2.1 ab	119.5 (114.4-130.3) a	4.0 (2.7-5.4) ab	6.0 (4-6) a
Non-inoculated	Primary	153.9 ± 10.7 a	13.0 ± 1.0 a	116.8 (113.8-128.6) a	5.0 (4.4-5.5) a	5.0 (5-6) a
	Tertiary	189.5 ± 16.2 ab	16.8 ± 1.5 ab	114.1 (108.1-118.4) a	4.4 (3.8-4.9) ab	6.0 (5-6) a
	Rainwater	242.7 ± 19.8 b	21.6 ± 1.8 b	113.4 (106.7-120.9) a	3.4 (3.0-3.7) b	5.0 (5-6) a

ANOVA	**df**	**F**	**p**	**F**	**p**	**H**	**p**	**H**	**p**	**H**	**p**
Inoculation	1	0.31	>0.05	0.51	>0.05	0.32	>0.05	1.54	>0.05	0.68	>0.05
Wastewater type	2	9.52	**<0.001**	8.97	**<0.001**	1.71	>0.05	22.49	**<0.001**	0.02	>0.05
Inoculation x wastewater type	2	1.46	>0.05	1.53	>0.05	3.26	>0.05	2.35	>0.05	0.46	>0.05

Values are means (±SE) unless otherwise stated. Significance levels for main effects and interactions are shown. ^{x} = median values (95% CI). Any samples in the same column with a common letter are not significantly different using the Tukey test (p > 0.05), or the Kruskal Wallis multiple comparison test (p > 0.05) where median values are shown.

Leaves on trees that received primary wastewater were significantly smaller and lighter-leaved than the leaves on trees that received tertiary wastewater or rainwater (Table 6.5). Inoculation did not have a significant effect on average leaf area or average leaf weight and there was no significant [inoculation x wastewater type] interaction.

For total leaf dry weight, trees that received primary wastewater had a significantly greater total leaf dry weight than did trees that received rainwater (Table 6.5). Inoculation did not have a significant effect on total leaf dry weight and there was no significant [inoculation x wastewater type] interaction. For both SLA and shoot number per plant there were no significant differences between treatments (Table 6.5). There was no difference in plant survival at the end of phase 1 (data not shown).

6.4.2 Phase 2 - development of a willow-selected mycorrhizal fungal inoculum

6.4.2.1 Ergosterol analysis

Ergosterol is a fungus-specific membrane sterol and can be used to quantify mycorrhizal fungi presence (Nylund and Wallander, 1992). Previously, measurement of ergosterol has been used to equalise mycorrhizal fungi isolates in terms of a common ergosterol density before inoculation onto host plants (Hart and Reader, 2002). The four treatments used in phase 2 were selected as the most likely treatments to exist at the time of ZDWS installation. No ergosterol was detected in either willow-selected DIEHARD rainwater irrigated roots or willow-selected resident mycorrhizal fungi rainwater irrigated roots (Table 6.6). Ergosterol concentration in willow-selected resident mycorrhizal fungi primary wastewater

irrigated roots were also low, with a number of replicates returning no detectable ergosterol. The highest ergosterol concentration was recorded in the willow-selected DIEHARD primary wastewater irrigated roots. Average ergosterol recovery rate from the root samples was 84% and in the basic DIEHARD the average ergosterol recovery rate was 90%.

Table 6.6 Mean ± SE ergosterol concentration in root inoculum and basic DIEHARD.

Inoculum	Ergosterol concentration (μg g^{-1} dry weight root or inoculum powder)
Willow-selected DIEHARD rainwater irrigated roots	0
Willow-selected resident mycorrhizal fungi rainwater irrigated roots	0
Willow-selected DIEHARD primary wastewater irrigated roots	11.9 ± 5.8
Willow-selected resident mycorrhizal fungi primary wastewater irrigated roots	0.6 ± 0.3
Basic DIEHARD inoculum	1.8 ± 0.2

6.4.2.2 Mycorrhizal colonisation

A significant difference in root mycorrhizal colonisation at the end of phase 2 was observed ($F(5,54) = 8.11$; $p < 0.001$) (Table 6.7). Trees inoculated with willow-selected DIEHARD primary wastewater irrigated roots and trees inoculated with willow-selected resident mycorrhizal fungi primary wastewater irrigated roots were found to have significantly higher mycorrhizal colonisation than trees inoculated with basic DIEHARD, trees inoculated with willow-selected resident mycorrhizal fungi rainwater irrigated roots and the control trees. The lowest level of mycorrhizal colonisation (7.4%) occurred for trees inoculated with willow-selected resident mycorrhizal fungi rainwater irrigated roots. This was significantly lower than the colonisation recorded for trees inoculated with willow-selected DIEHARD rainwater irrigated roots.

Table 6.7 Mean ± SE mycorrhizal colonisation of willow roots at the end of phase 2. Any samples which share a common letter are not significantly different using the Tukey test (p > 0.05).

Treatment	Colonisation (%)
Willow-selected DIEHARD rainwater irrigated roots	25.2 ± 5.0 bc
Willow-selected resident mycorrhizal fungi rainwater irrigated roots	7.4 ± 6.7 a
Willow-selected DIEHARD primary wastewater irrigated roots	33.5 ± 4.2 c
Willow-selected resident mycorrhizal fungi primary wastewater irrigated roots	28.7 ± 4.3 c
Basic DIEHARD inoculum	21.2 ± 3.8 ab
Control	12.6 ± 2.2 ab

6.4.2.3 Tree growth

Differences in shoot height between the six treatments began to emerge after seven weeks (Figure 6.4). At the end of the trial, significant differences were observed ($F(5,59) = 26.702$; $p < 0.001$) (Figure 6.4 and Figure 6.5). Trees inoculated with willow-selected DIEHARD rainwater irrigated roots or willow-selected DIEHARD primary wastewater irrigated roots were significantly taller than trees inoculated with willow-selected resident mycorrhizal fungi rainwater irrigated roots, willow-selected resident mycorrhizal fungi primary wastewater irrigated roots and the control trees (Figure 6.4). The final shoot heights of the former two treatments were taller than the trees inoculated with basic DIEHARD, but this difference was not significant. Trees inoculated with willow-selected resident mycorrhizal fungi primary wastewater irrigated roots and willow-selected resident mycorrhizal fungi rainwater irrigated roots reached a final shoot height similar to that of the control trees (Figure 6.4).

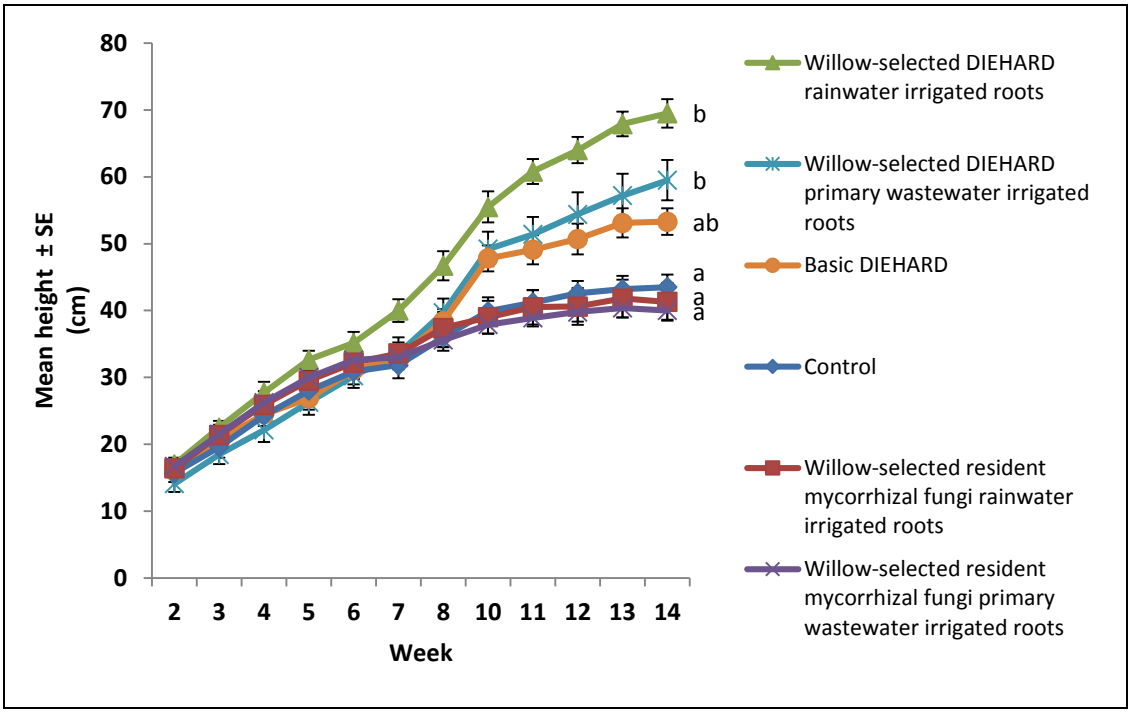


Figure 6.4 Mean ± SE shoot height of leading stem during phase 2. Any week 14 samples which share a common letter are not significantly different using the Tukey test (p > 0.05).

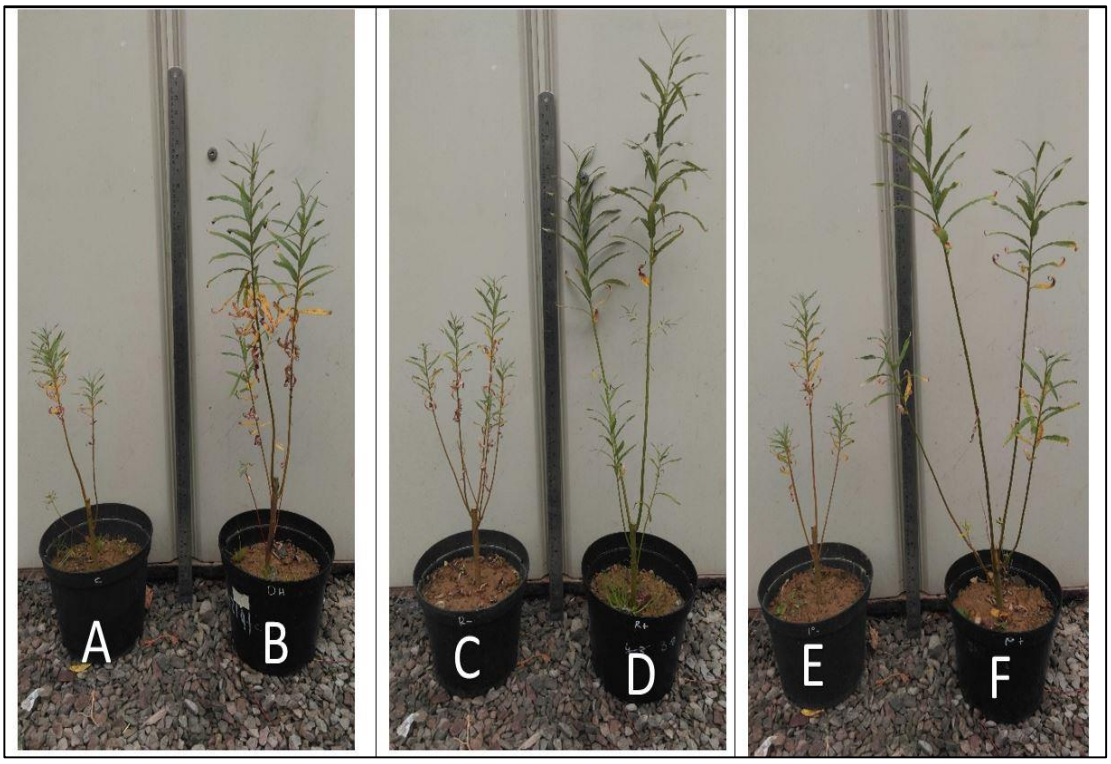
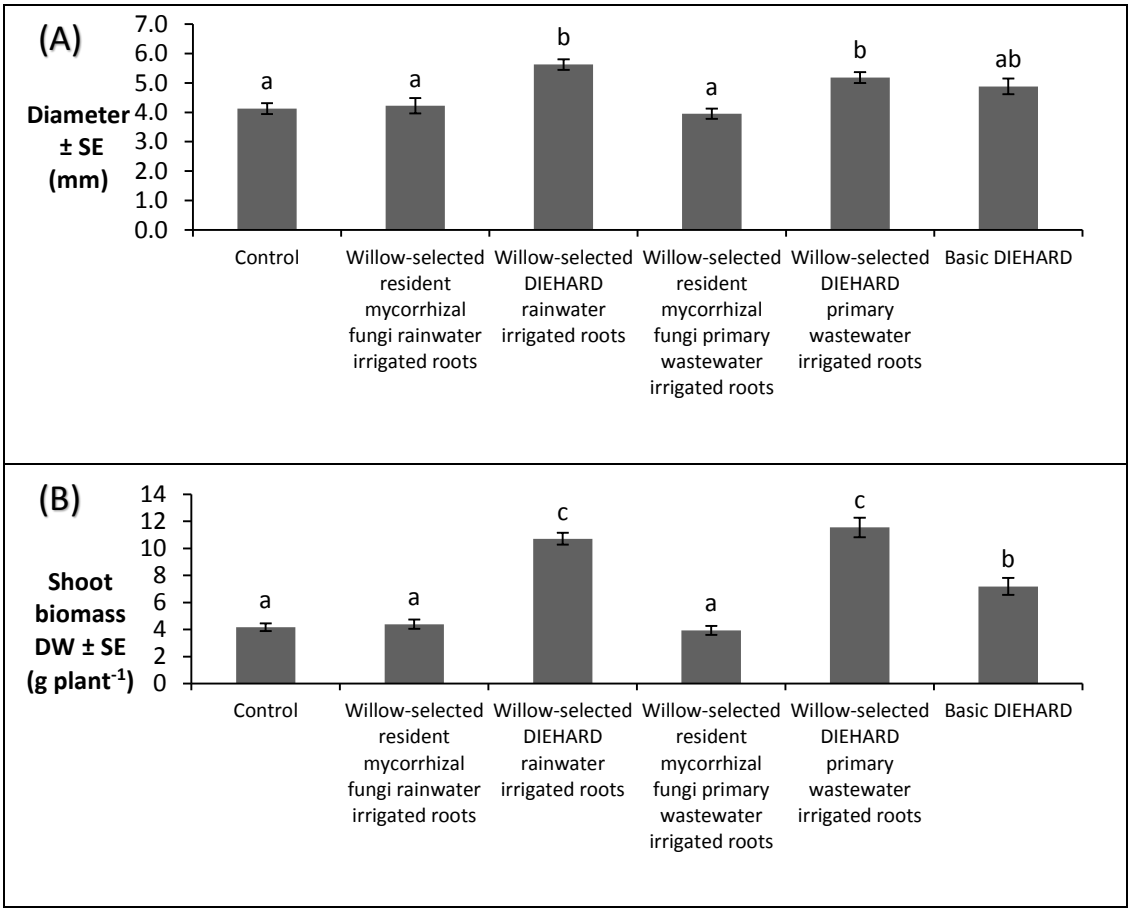


Figure 6.5 Examples of phase 2 trees before harvest. (A) Control, (B) Basic DIEHARD, (C) Willow-selected resident mycorrhizal fungi rainwater irrigated roots, (D) willow-selected DIEHARD rainwater irrigated roots, (E) willow-selected resident mycorrhizal fungi primary wastewater irrigated roots, (F) willow-selected DIEHARD primary wastewater irrigated roots. Metal bar in centre of images = 1 m.

A similar trend occurred for diameter, with significant differences observed ($F(5,59) = 7.65$; $p < 0.001$) (Figure 6.6). Trees inoculated with willow-selected DIEHARD rainwater irrigated roots and willow-selected DIEHARD primary wastewater irrigated roots had significantly broader diameters than trees inoculated with either willow-selected resident mycorrhizal fungi rainwater irrigated roots, willow-selected resident mycorrhizal fungi primary wastewater irrigated roots, or the control trees (Figure 6.6). Tree diameters from the former two treatments were also broader than the trees inoculated with basic DIEHARD, but this difference was not significant. Trees inoculated with willow-selected resident mycorrhizal fungi primary wastewater irrigated roots and willow-selected resident mycorrhizal fungi rainwater irrigated roots reached a final shoot diameter similar to the control trees.

Significant differences were also observed for shoot biomass ($F(5,59) = 50.24$; $p < 0.001$). Trees inoculated with willow-selected DIEHARD rainwater irrigated roots and willow-selected DIEHARD primary wastewater irrigated roots had the greatest shoot biomass, which was significantly greater than trees inoculated with basic DIEHARD. Trees inoculated with basic DIEHARD were significantly heavier than trees inoculated with willow-selected resident mycorrhizal fungi primary wastewater irrigated roots, willow-selected resident mycorrhizal fungi rainwater irrigated roots and the control trees (Figure 6.6).

Figure 6.6 Mean ± SE final shoot diameter (A) and shoot DW biomass (B) at the end of phase 2. Any samples which share a common letter are not significantly different using the Tukey test (p > 0.05).



Significant differences in average leaf area were observed ($F_{(5,50)} = 24.29$; $p < 0.001$)(Table 6.8). Leaves of trees inoculated with willow-selected DIEHARD primary wastewater irrigated roots, willow-selected DIEHARD rainwater irrigated roots or basic DIEHARD roots were significantly larger than the leaves from the other three treatments.

Table 6.8 Mean ± SE final leaf parameters and shoot growth parameters at end of phase 2. Any samples within a column which share a common letter are not significantly different using the Tukey test ($p > 0.05$).

Treatment	Average leaf area (mm^2)	Average leaf dry weight (mg)	SLA ($cm^2\ g^{-1}$)	Total tree leaf area (cm^2)	Total tree leaf dry weight (g)	No. shoots $plant^{-1}$
Control	166.8 ± 8.0 a	13.4 ± 0.6 a	125.0 ± 3.4 a	140.6 ± 13.5 a	1.1 ± 0.1 a	5.4 ± 0.6 a
Willow-selected resident mycorrhizal fungi rainwater irrigated roots	158.1 ± 8.8 a	12.5 ± 0.9 a	131.5 ± 12.6 a	157.6 ± 14.5 a	1.2 ± 0.1 a	5.8 ± 0.6 a
Willow-selected DIEHARD rainwater irrigated roots	302.7 ± 20.7 b	23.0 ± 2.3 b	137.0 ± 8.7 a	531.8 ± 43.7 c	4.0 ± 0.2 c	4.8 ± 0.4 a
Willow-selected resident mycorrhizal fungi primary wastewater irrigated roots	144.8 ± 8.2 a	11.4 ± 0.3 a	126.6 ± 5.5 a	135.8 ± 11.4 a	1.1 ± 0.1 a	5.2 ± 0.3 a
Willow-selected DIEHARD primary wastewater irrigated roots	335.1 ± 31.6 b	26.5 ± 2.8 b	127.8 ± 2.7 a	596.4 ± 62.2 c	4.7 ± 0.5 c	6.4 ± 0.6 a
Basic DIEHARD	307.9 ± 44.1 b	24.9 ± 2.3 b	124.6 ± 2.4 a	297.9 ± 43.2 b	2.4 ± 0.3 b	5.2 ± 0.4 a

A similar trend was observed with respect to average leaf weight ($F(5,50) = 18.60$; $p < 0.001$)(Table 6.8). Leaves of trees inoculated with willow-selected DIEHARD primary wastewater irrigated roots, willow-selected DIEHARD rainwater irrigated roots and basic DIEHARD roots were significantly heavier than the leaves from the other three treatments.

Significant differences were observed for total tree leaf dry weight ($F(5,50) = 34.52$; $p < 0.001$) and total tree leaf area ($F(5,50) = 37.55$; $p < 0.01$ (Table 6.8). Trees inoculated with willow-selected DIEHARD primary wastewater irrigated roots and willow-selected DIEHARD rainwater irrigated roots had a significantly larger total tree leaf dry weight and total tree leaf area than did trees inoculated with basic DIEHARD. Trees inoculated with basic DIEHARD had a significantly larger total tree leaf dry weight and total tree leaf area than did trees inoculated with willow-selected resident mycorrhizal fungi primary wastewater irrigated roots, willow-selected resident mycorrhizal fungi rainwater irrigated roots or the control trees.

No significant differences in final shoot number or SLA between the different treatments were found at the end of phase 2 (Table 6.8). There were no significant differences in plant survival between any of the six treatments (data not shown).

6.4.3 Phase 3 - qPCR analysis

The qPCR markers used here proved to be specific for the different mycorrhizal species; no cross-reactivity was detected with related or unrelated species, with the exception of *Glomus etunicatum* and *G. claroideum*, which could not be distinguished. As a result, the estimates for *G. claroideum* and *G. etunicatum* were pooled (Table 6.9). Broadly, these nLSU markers are well suited to the identification and quantification of the target mycorrhizal fungal species in fungal communities.

Table 6.9 Quantification of willow root colonisation by different mycorrhizal fungal taxa, using qPCR primers for nLSU of ribosomal DNA. Any samples within a column with a common letter are not significantly different ($p > 0.05$) using the Kruskal-Wallis test.

Species	Mean ng genomic DNA mg^{-1} root dry weight		Mean ng genomic DNA mg^{-1} powder
	Resident willow inoculum	Willow-selected inoculum	Basic DIEHARD inoculum
AMF Species			
Glomus mosseae	1.8 abc	16.7 ab	9.7 c
G. intraradices	0.5 abc	20.2 a	11.5 c
G. claroideum/etunicatum	0.2 bc	9.8 bc	28.1 b
G. clarum	1.5 ab	1.7 c	11.6 c
G. microaggregatum	0.0 c	0.2 c	10.8 c
ECM Species			
Pisolithus tinctorius	2.0 a	5.8 c	257.7 a
Rhizopogon occidentalis	2.6 ab	6.1 c	27.2 b

The amount of each AMF isolate in the commercial inoculum was the same (Table 6.2). Quantification of the target species in basic DIEHARD by qPCR showed that no significant difference was detected in the amount of each AMF species in the basic DIEHARD inoculum, with the exception of the *G. claroideum/G. etunicatum* pooled sample (Table 6.9), which gave a value approximately twice that of the other AMF species, as expected. As a result, the qPCR estimates agreed closely with the

amounts listed in the inoculum. With respect to the ECM, the proportion of *P. tinctorius* to *R. occidentalis* were approximately 6:1 in the inoculum (Table 6.2) but closer to 10:1 according to the qPCR estimates (Table 6.9), suggesting a slight difference in sensitivity of the qPCR primers between the two ECM species.

The percentage mycorrhizal colonisation, quantified by microscopy studies, of willow roots from inoculated and non-inoculated trees (Table 6.4) were not significantly different from one another. As the experiments were carried out in non-sterile soil to approximate field conditions, the non-inoculated roots had presumably been colonised by resident mycorrhizal isolates.

Although the amounts of each mycorrhizal fungal species in the DIEHARD inoculum (weight of propagules/unit weight of inoculum) and in the willow root inocula (weight gDNA/root weight) are in different units, differences in the proportion of the different species compared to the basic DIEHARD inoculum can identify changes in the species composition. Of the mycorrhizal fungal species present in the DIEHARD inoculum, all were also present in the willow-selected root inoculum (Table 6.9). When the amount of each AMF species in the willow-selected inoculum was assessed relative to that of *G. intraradices*, it was clear that *G. mosseae* was as frequent as *G. intraradices*, higher than *G. etunicatum*/*G. claroideum* and much higher than *G. microaggregatum* and *G. clarum* indicating that selection by colonisation of the willow roots had markedly changed the species composition of the inoculum, compared to that of the DIEHARD inoculum. Furthermore, the relative proportion of *P. tinctorius* to *R. occidentalis* had also changed

The resident population contained all the AMF and ECM species present in the DIEHARD inoculum, with the exception of *G. microaggregatum*. This is not surprising given the widespread distribution of these species. The amounts of each species in the non-inoculated roots, however, were much smaller than those of the same species in the inoculated roots. Given that the colonisation percentages were similar in the two sets of roots, these figures indicate that other mycorrhizal species make up the majority of the mycorrhizal colonisation in the non-inoculated roots.

6.5 Discussion

In this trial, phase 1 assessed the response of *S. viminalis* cuttings to a commercial mixed-species inoculum (DIEHARD^{TM} Transplant), in the presence of three different wastewater types and two different irrigation rates. Phase 2 examined the potential for developing a willow-selected mycorrhizal inoculum from a commercial mixed-species inoculum**.** Phase 3 used qPCR to identify which mycorrhizal fungi species were selected for in the willow-selected mycorrhizal inoculum

Phase 1 - response to commercial mycorrhizal fungal inoculum

Colonisation levels in both the inoculated and non-inoculated trees were similar, indicating colonisation of the roots from the resident mycorrhizal population in the soil. Colonisation levels were similar to those found on willows by Hashimoto and Higuchi (2003), van der Heijden and Kuyper (2003) and Püttsepp *et al.* (2004), but higher than those found by Trowbridge and Jumpponen (2004) and Fillion *et al.* (2011).

According to Rooney *et al.* (2009), no clear relationship has been found between nutrient application rate and mycorrhizal colonisation in willow SRC. In the current

study, wastewater type did not have a significant effect on mycorrhizal colonisation, which agrees with the results found by Quoreshi and Khasa (2008) in poplar. Previous studies have found both increases and decreases in mycorrhizal colonisation levels in the presence of nutrient application (Jones *et al.*, 1990; Baum and Makeschin, 2000; Fillion *et al.*, 2011). The relationship between nutrient application and mycorrhiza colonisation is complex and dependent on soil properties such as texture, soil N and P content, soil pH, as well as site-specific factors such as climate and site management (Baum *et al.*, 2002a).

Increased growth by mycorrhiza-inoculated trees compared to non-inoculated trees has been found for willows (Jones *et al*., 1991; van der Heijden and Kuyper, 2003; Fillion *et al*., 2011), poplars (Baum *et al.*, 2002b; Quoreshi and Khasa, 2008) and eucalyptus (Arriagada *et al.*, 2009). In the current study, tree height, leading shoot diameter and shoot biomass, were all significantly larger in DIEHARD-inoculated trees compared to non-inoculated trees, but only when irrigated with rainwater. The significant positive effect on tree growth of DIEHARD inoculation for trees receiving rainwater indicates that increased growth only occurred in the relatively nutrient-poor environment of the rainwater treatment. Hence, inoculation should be beneficial in the start-up phase of a ZDWS i.e. where a ZDWS is installed before the house is occupied and the willows are rain-fed. Inoculation did not lead to significant difference in diameter or shoot biomass for trees receiving primary wastewater or tertiary wastewater, i.e. relatively nutrient-rich environments, suggesting that mycorrhizal colonisation aids recovery of nutrient from soil. This

indicates that inoculation may not be necessary if a ZDWS is to be installed to receive wastewater in the first year.

As mycorrhizal colonisation levels were similar at the end of phase 1, yet with significantly different plant responses in the case of trees receiving rainwater, these results agree with those of van der Heijden and Kuyper (2003) who found that differences in plant growth have been shown to be unrelated to colonisation level in *S. repens*. Instead, the quality i.e. mycorrhizal fungi identity, rather than the quantity of fungal colonisation, can control the size and direction of the plant growth response to the presence of specific mycorrhiza (Baum *et al.*, 2009; Fransson *et al.*, 2013). In the case of the trees receiving rainwater, clearly the presence of the introduced mycorrhizal species conferred an advantage to these trees which was absent in the presence of the native soil mycorrhizal population.

DIEHARD-inoculated trees, which received rainwater, had similar levels of growth to non-inoculated trees that received tertiary wastewater in both height and diameter measurements. For these two parameters, this result indicates that the presence of the commercial mycorrhizal inoculum was as effective as nutrient application through tertiary wastewater application.

No significant differences in average leaf area, average leaf dry weight or SLA between inoculated and non-inoculated trees were found. This contrasts with the findings of Amerian *et al.* (2001), who found that inoculation with *G. mosseae* or *G. intraradices* led to a higher average leaf area and SLA compared to non-inoculated maize plants. A higher SLA can lead to benefits to plant growth as the amount of

photosynthetically active radiation that can be captured on a per unit leaf mass basis is increased (Tholen *et al.*, 2004).

Total tree leaf weight was greatest in trees that received primary wastewater. Similar results were reported by Lower *et al.* (2003) who found that nutrient application led to an increase in total willow leaf weight. However, average leaf area and average leaf weight were significantly smaller in trees that received primary wastewater. This is in contrast with the results of other studies (Heilman and Fu-Guang, 1994; Lower *et al.*, 2003; Isebrands and Richardson, 2014) who found that nutrient application has been shown to lead to a larger leaf area in willows. As total willow leaf weight was greatest in trees irrigated with primary wastewater, this indicates that the total leaf number of the primary wastewater trees was higher, which was most likely due to the larger tree size, as reflected in the height, diameter and shoot biomass results.

Irrigation with landfill leachate can reduce willow leaf area due to toxic effects of the leachate (Dimitriou *et al.*, 2006). Toxicity effects were unlikely to have occurred in this trial as primary wastewater has been shown to be highly suitable for willow irrigation (Perttu and Kowalik, 1997; Curneen and Gill, 2014). Instead, it is likely that at the time of harvest, trees that received primary wastewater may have been water stressed due to the large size of the trees and the relatively small size of the pots. Under water-limiting conditions, willows reduce individual leaf area (Weih *et al.*, 2011), which may account for the lower individual leaf area (and hence individual leaf mass) results here.

Phase 2 - development of a willow-selected mycorrhizal fungal inoculum

In step 1 of phase 2, an ergosterol assay was carried out to quantify and equalise the mycorrhizal fungi amounts in different sources of mycorrhizal inoculum. Ergosterol recovery rates were similar to the recovery rates found by Salmanowicz and Nylund (1988). The root ergosterol concentration of the trees irrigated with primary wastewater was lower than the concentrations found by Salmanowicz and Nylund (1988) in mycorrhized pine roots, but similar to the levels found by Olsrud *et al.* (2007) in subarctic shrubs, and also similar to the levels found by Hart and Reader (2002) for *Plantago lanceolota* L., *Plantago major* L., *Poa pratensis* L. and *Poa annua* L.

In contrast, no ergosterol was detected in the roots of trees that received rainwater. The absence of detectable ergosterol in the rainwater roots may have been due to a number of reasons. Ergosterol concentrations in the rainwater roots may simply have been markedly lower than the primary wastewater roots. The technique of Mohd As'wad *et al.* (2011) has been used successfully in the past to detect fungal presence in oil palm sections. However, to increase the amount of ergosterol that can be detected, the addition of a saponification step to the protocol, as used by Salmanowicz and Nylund (1988), should be considered as saponification has been shown to purify the sample (Nylund and Wallander, 1992). Another possible explanation may be based on the fact that the ergosterol assay detects only actively growing mycorrhizal fungi (Ekblad *et al.*, 1998). The mycorrhizal fungi in the roots of trees exposed to the rainwater treatments may not have been actively growing at the time of sampling. Similarly, the very low

detectable amount of ergosterol found in the basic DIEHARD may have been due to the fact that the mycorrhizal fungi in this commercial inoculum were inactive.

Step 2 of phase 2 examined the potential for developing a willow-selected mycorrhizal inoculum from a commercial mixed-species inoculum**.** Mycorrhizal colonisation levels were lower than the levels recorded during phase 1, but similar to levels recorded by Fillion *et al.* (2011). Trees inoculated with willow-selected DIEHARD primary wastewater irrigated roots and willow-selected resident mycorrhizal fungi primary wastewater irrigated roots had higher levels of mycorrhizal colonisation than did control trees or trees that received basic DIEHARD. This is in contrast to phase 1, where there was no difference in colonisation between control trees and DIEHARD inoculated trees. This finding agrees with that of Cooper (1987) and Klironomos and Hart (2002), whereby infected roots are known to be an effective source of mycorrhizal inoculum. The results of the current study supports the hypothesis that the use of willow-selected DIEHARD root inoculum could increase the percentage mycorrhizal colonisation compared to a broad-spectrum commercial inoculum, such as DIEHARD.

While significant differences in mycorrhizal colonisation were recorded at the end of phase 2, a similar trend was observed as in phase 1, with no clear relationships between level of colonisation and plant response. Trees inoculated with willow-selected resident mycorrhizal fungi primary wastewater irrigated roots had the second highest levels of mycorrhizal colonisation, yet shoot biomass production was similar to that of control trees. Control trees and trees inoculated with basic DIEHARD had similar levels of mycorrhizal colonisation, yet growth of the basic

DIEHARD trees was significantly larger. Low mycorrhizal colonisation rates does not mean low plant response (van der Heijden, 2001), and hence, as in phase 1, and as found by Baum *et al.* (2009) it is the quality, rather than the quantity, of the colonisation (in terms of fungal spp. present) which determined plant response.

There were no differences in plant growth parameters between willow-selected resident mycorrhizal fungi primary wastewater irrigated roots, willow-selected resident mycorrhizal fungi rainwater irrigated roots and control trees in any measured plant parameter. In contrast, trees inoculated with willow-selected DIEHARD primary wastewater irrigated roots or willow-selected DIEHARD rainwater irrigated roots were significantly larger than trees inoculated with willow-selected resident mycorrhizal fungi primary wastewater irrigated roots, willow-selected resident mycorrhizal fungi rainwater irrigated roots or control trees in all plant growth measurements except for SLA and final shoot number. For biomass, there was a 1.5 to 1.9 fold increase between trees that received willow selected DIEHARD roots and trees that received willow-selected resident mycorrhizal fungi roots and the control trees. This indicated that selection of certain species from the commercial DIEHARD had taken place.

A general trend occurred whereby trees inoculated with willow-selected DIEHARD primary wastewater irrigated roots or willow-selected DIEHARD rainwater irrigated roots were larger than trees that received basic DIEHARD (significant increases in shoot biomass, total tree leaf weight and total tree leaf area). Increased shoot biomass and increased leaf area led to higher evapotranspiration rates in willow (Persson, 1995; Curneen and Gill, 2014). Thus, the creation of a willow-selected

inoculum for ZDWS, to maximise willow shoot biomass production and willow leaf area, should lead to increased evapotranspiration rates.

The enhanced performance of trees inoculated with willow-selected DIEHARD primary wastewater irrigated roots or willow-selected DIEHARD rainwater irrigated roots in comparison to the basic DIEHARD indicates that some selection had taken place during phase 1. Selection factors were likely to have included the willow variety, soil type and nutrient application conditions, as these conditions are known to influence the level of mycorrhizal colonisation and the impact of mycorrhizal inoculation on willow aboveground biomass production (Hrynkiewicz *et al.*, 2012; Wijesinghe, 2012; Fransson *et al.*, 2013). These results agree with both Molina *et al.* (1992) and Ortega *et al.* (2004), whereby the use of inocula that are adapted to certain hosts or habitats has the potential to be more beneficial than the development of broad-spectrum inocula. Thus, the inoculation of these willow-specific inocula, that are adapted to both host (the *Salix viminalis* clone used in this study) and habitat conditions (heavy clay soil, rainwater/primary wastewater application), has been demonstrated to show promise.

Wastewater type was not a factor in phase 2, with all trees receiving rainwater only. The improved performance of trees inoculated with willow-selected DIEHARD primary wastewater irrigated roots or willow-selected DIEHARD rainwater irrigated roots must be interpreted in this light. If the improved performance of these trees was due to an increased ability to take up nutrients from the soil, this may only be the case when trees are growing in a nutrient-poor environment i.e. rainwater fed. In phase 1, it was found that when grown in a nutrient-rich environment (i.e.

wastewater-fed), there were no significant differences in shoot biomass growth between DIEHARD inoculated and non-inoculated trees. Hence, the benefit of the "willow-selected" inoculum may be limited to the start-up phase of a ZDWS only when trees are rainwater-fed.

Phase 3 - qPCR analysis

The analysis of the mycorrhizal species composition in the inoculated and non-inoculated roots showed that the qPCR method provides a very useful proxy (Gamper *et al*., 2008) for the abundance of individual mycorrhizal fungal species in the analysis of mixed-species mycorrhizal populations. This strategy has become well adopted by the mycorrhizal research community (Kennedy *et al*., 2007; Pivato *et al*., 2007; Thonar *et al*., 2012).

The species used in DIEHARD have been demonstrated to increase performance of inoculated host plants (e.g. Stamets (2005)), and in this study changes in AMF and ECM relative proportions were associated with the increased crop performance of inoculated willow. For AMF, only certain *Glomus* species increased as a result of selection. Jansa *et al.* (2002) reported that *Glomus* species were particularly disturbance tolerant, being adapted to, say, tillage conditions. During the construction and establishment of a ZDWS (or the growth studies described earlier in the chapter), tolerance of disturbance would be useful. Selection pressures could change in the later stages when the conditions become more stable, so further studies on mycorrhizal selection could profitably be carried out once the establishment phase has been completed as different AMF and ECM species can lead to different benefits depending on the stage of willow growth (Jones *et al*.,

1991; van der Heijden, 2001; Fillion *et al*., 2011). The ECM species used in this trial are known to be affected by a range of conditions including soil moisture and plant host (Kennedy and Peay, 2007; Quoreshi and Khasa 2008) which may have resulted in the observed change in relative proportions. Only a limited number of mycorrhizal fungal species were included in this study. It would be valuable to widen the number of species being used in further selection studies as willows are known to be dual mycorrhizal and capable of associating with a number of different mycorrhizal species (Trowbridge and Jumpponen, 2004).

This is the first reported use of the qPCR protocol to quantify the changes in species composition during the process of selection for ability to colonise the root system of a particular host plant. Although the ability to colonise a root system and to confer a stimulant effect are not necessarily causally related, in this case, the willow-selected inoculum, with increases in relative abundance of the AMF *Glomus mosseae* and *G. intraradices*, showed significant increases in willow growth parameters.

6.6 References

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Chapter 7 Conclusions and perspectives

Background to the research

Wastewater contains a wide range of contaminants including high levels of nutrients, parasites, and pathogens (Macler and Merkle, 2000; Bitton, 2005). In the absence of centralised wastewater treatment systems in rural areas, protection of surface waters and groundwater is dependent on OWTS. Where these systems do not operate effectively, pollution of waters and groundwater can occur, threatening both human health and the environment (EPA, 2015).

OWTS typically consist of a septic tank, followed by a percolation area or packaged treatment system. In the traditional septic tank and percolation area system, a critical part of the on-site wastewater treatment process is the ability of the soil to receive the hydraulic loading of the effluent, as the majority of the wastewater treatment takes place in the first 0.6 m below the percolation area pipes (Gill *et al*., 2007). Where conditions of low subsoil permeability occur, such that the subsoil cannot accept the hydraulic loading, surface ponding can occur (McCarthy *et al*., 2009). Across Ireland, 39% of all sites will experience inadequate percolation for all or part of the year (EPA, 2012). For a low permeability site where the percolation test is failed, the site is deemed unsuitable for wastewater discharge to ground (EPA, 2009). As discharge of domestic wastewater to surface waters is not favoured by local authorities (Dubber and Gill, 2014), there is a pressing need to develop a system for sites where the percolation test is failed.

The use of a ZDWS offers potential as a solution to these sites of low subsoil permeability (Brix and Arias, 2011). However, the very large size of these conventional ZDWS (125 m^2 per person in Ireland)(Curneen and Gill, 2015) and the

high installation costs (€5,300 per capita)(Dubber and Gill, 2014) can limit their practical use. Reducing the area demand of a ZDWS, through increased efficiency of wastewater evapotranspiration, may help in increasing the use of ZDWS in low permeability sites.

The aim of this study was to examine the development of a novel ZDWS, for use for domestic wastewater treatment in rural Ireland. More specifically, the objectives were: (i) to investigate the use of polytunnel covered ZDWS to increase wastewater removal from a ZDWS by eliminating ingress from rainfall; (ii) to examine the potential use of a winter-active species, *P. fragrans,* to increase evapotranspiration in a ZDWS; (iii) to investigate the impact of annual coppicing and a gradient in soil clay percentage on willow evapotranspiration, growth and nutrient balance; (iv) to investigate the effect of commercial mixed-species mycorrhizal fungal inoculum on the growth of *Salix viminalis* trees; (v) to explore the development of a willow-specific mycorrhizal fungal inoculum for use in ZDWS. The following sections discuss the main conclusions of the study, including some limitations of the research. Recommendations for the management of these systems are made, alongside identification of areas for future research.

The development of a novel covered system

In Denmark, 50 - 80% of the water lost from a ZDWS through evapotranspiration entered the system as rainfall (Ministry of Environment and Energy, 2001). The large input of rainfall necessitates that the ZDWS has adequate storage capacity to accommodate all of the hydraulic loading (Gregersen and Brix, 2001). This requirement for water storage contributes to the very large size of these systems,

which can restrict the use of these systems on certain sites due to land availability constraints (Dubber *et al*., 2014). Previously, Curneen and Gill (2015) showed that ZDWS in Ireland would not operate on a true zero-discharge basis due to the high levels of rainfall in Ireland. The current study has developed a true zero-discharge system by the placement of a polytunnel cover to remove 100% of the incident rainfall. Evapotranspiration rates from the [+ willow + cover] wetlands of 665 - 828 mm per year were similar to willow evapotranspiration rates reported from elsewhere in Ireland (Curneen and Gill, 2014, 2015). However, in terms of wastewater removal efficiency, i.e. area demand per person, the covered system was shown to be more efficient at wastewater removal than the ZDWS of Curneen and Gill (2015) and as efficient as ZDWS in Denmark (Ministry of Environment and Energy, 2003)(Table 3.6).

The importance of the polytunnel cover was further highlighted in the lysimeter study (section 5.4.1). During the dry summer of 2014 (292 mm rainfall), an average of 771 mm of wastewater was applied to the coppiced trees, and an average of 806 mm of wastewater was applied to the non-coppiced trees. In comparison, during the wet summer of 2015 (519 mm rainfall), 336 mm of wastewater was applied to the coppiced trees and 412 mm to the non-coppiced trees. This indicates that the difference in wastewater removal between a wet and a dry summer could be as large as 400 mm. Due to the recent rise in global mean temperatures and global climate change, increased precipitation has occurred along with an increase in the frequency of heavy precipitation events (Steele-Dunne *et al*., 2008). In light of this

increased rainfall loading to the system, the use of a cover appears to increase the appeal of these systems for use in Ireland.

No significant difference in annual evapotranspiration was found between the [+ willow + cover] wetland type and the [- willow + cover] wetland type (section 3.4.2). This indicates that an unplanted covered system could be considered in place of the traditional tree-based ZDWS. The high levels of evaporation from the unplanted system was attributed to the application of wastewater to the wetland surface, as opposed to the subsurface, which would maximise evaporation from the wet soil. An advantage of the unplanted system is the reduced installation costs (no requirement to purchase, store and plant trees) and maintenance costs (no requirement for pest/pathogen management or annual coppicing) that would occur in the absence of the need for willow trees. Furthermore, as willow yield in the [+ willow + cover] wetlands declined from 2011 to 2013 (section 3.4.4) due to annual coppicing, the use of a system that does not depend on willow yield would appear to be advantageous.

In contrast to the declining willow yield recorded in the Cloyne study, no decline in willow yield was found for the annually coppiced willows in the lysimeter study (section 5.4.2). However, willow yield was only measured across two seasons, in comparison to the Cloyne study, where yield was measured across four years. Successive annual coppicing is known to affect willow yield (Kopp *et al*., 1997) and willow yield in the annually coppiced lysimeters may have declined if the measurement period had been extended to three or four years.

A limitation of this study was that the method of wastewater application was "demand lead" i.e. wastewater was only applied when the water level in the wetland was low. This allowed for increased wastewater application during the summer and lower application during the winter months (Figure 3.9). In contrast, in a fully functioning ZDWS connected to a house, wastewater application would be "supply lead" i.e. where wastewater would be supplied to the ZDWS, irrespective of whether the wetland had sufficient capacity. Wastewater production from a house is reasonably consistent throughout the course of a year (Ministry of Environment and Energy, 2003). This constant supply of wastewater over the course of a year may lead to conditions of sub-maximum evapotranspiration during the summer due to inadequate water supply. During the winter, should a covered ZDWS reach capacity, wastewater may need to be diverted to a storage container for application during the summer months. Thus, the area requirement figures in Table 3.6 should be interpreted with this in mind.

A gradient in soil clay percentage from 5% to 30% did not lead to any significant differences in willow yield or evapotranspiration from annually coppiced trees (sections 5.4.1 and 5.4.2). As the soils used in this study were of a similar clay percentage to the clay percentages used in ZDWS (up to 20% clay) (Ministry of Environment and Energy, 2003) this indicates that, for soils within this percentage clay gradient, the effect of soil type on evapotranspiration does not need to be factored into the water balance calculations used at the system design stage.

The use of a winter-active species to increase winter evapotranspiration

Investigations into the use of the winter-active species, *P. fragrans,* to increase winter evapotranspiration found that this species is not suitable for this purpose. This was mainly due to the low levels of winter evapotranspiration, which were very similar to levels from unplanted soil, and also the inability of the species to tolerate even short duration waterlogging events (sections 4.4.2 and 4.4.3). In addition, intercropping conferred a disadvantage on aboveground and belowground biomass production for both *S. viminalis* and *P. fragrans* (section 4.4.4).

An alternative winter-active species to increase winter evapotranspiration may not be required. In chapter 3, the placement of a cover in unplanted wetlands increased annual wastewater removal efficiency to a level that is more efficient than traditional non-covered ZDWS (Table 3.6). The contribution of soil evaporation to the annual evapotranspiration was large, as shown by the high evaporation levels from the wet soil of the [- willow + cover wetlands](section 3.4.2) and the low evapotranspiration/evaporation ratio (section 3.5). Furthermore, the crop factor of the [- willow + cover] wetland type frequently ranged from 1.5 to 2.0 during the winter months (Figure 3.10) which was slightly above the *P. fragrans* crop factors recorded during the evapotranspiration trial (Figure 4.7). This indicates that the presence of the polytunnel is more effective at raising winter evaporation levels than *P. fragrans.*

The development and evaluation of a willow-selected mycorrhizal inoculum

Good establishment and first year growth is crucial to the long-term success of ZDWS (Curneen and Gill, 2012). This study has shown that the use of a commercial

mycorrhizal fungi inoculum (DIEHARD) can increase willow biomass growth in the establishment stage of a ZDWS (section 6.4.1). To date, no clear trend is apparent from the literature of the effect of mycorrhizal fungal inoculation on willow growth, with previous studies showing varying willow growth responses to inoculation (Jones *et al*., 1990; van der Heijden, 2001; Baum *et al*., 2009).

For willows irrigated with rainwater, those that were inoculated with commercially available DIEHARD had a significantly larger biomass than rainwater irrigated control willows that were not inoculated. While inoculated and non-inoculated trees that received primary or tertiary wastewater had a significantly larger biomass than inoculated or non-inoculated trees that received rainwater, nutrient application to willow SRC during the first year is generally not advised as it can exacerbate weed problems (Caslin *et al*., 2010) and lead to negative effects on willow yield due to the accumulation of standing water on the surface of the ZDWS (Ministry of Environment and Energy, 2001). Thus, where a ZDWS is installed prior to home occupation, and as a result is rainwater fed, the use of a commercial mixed-species mycorrhizal inoculum can increase biomass growth relative to non-inoculated trees.

In this study, increased mycorrhizal fungal colonisation did not lead to increased willow growth (sections 6.4.1 and 6.4.2) which was in agreement with the findings of van der Heijden (2001) and van der Heijden and Kuyper (2003). Thus, the identity, i.e. quality rather than quantity, of the mycorrhizal species was found to play an important role in plant response. Furthermore, this study found that the development of a willow-selected mycorrhizal inoculum for use in ZDWS shows

promise, with significantly higher biomass growth in trees inoculated with willow-selected DIEHARD primary wastewater irrigated roots and willow-selected DIEHARD rainwater irrigated roots compared to trees colonised by the resident soil mycorrhizal fungi (section 6.4.2). Analysis of the changes in mycorrhizal species abundance associated with willow roots highlighted the importance of the disturbance-tolerant *Glomus mosseae* and *G. intraradices* (Section 6.4.3). This finding agrees with that of Molina *et al.* (1992), whereby the development of host-specific and habitat-specific mycorrhizal fungal inocula is regarded as more effective than the creation of broad-spectrum, generic mycorrhizal fungal inocula.

The application of this research can be extended beyond ZDWS, and into traditional willow SRC plantations, whereby the use of mycorrhizal species that are best adapted to willow species and site factors can be explored. Mycorrhizal fungi colonisation is known to be affected by soil disturbance (Lekberg and Koide, 2005) and the use of mycorrhizal inocula in SRC may be quite advantageous, due to the perennial nature of the crop and the lack of mycorrhizal disturbance of the root system over the life-cycle of the SRC plantation (Rooney *et al*., 2009).

A limitation of the mycorrhizal studies was that the effect of flooded conditions on the mycorrhiza was not explored. Pots were free-draining and flooded conditions did not occur. Where wastewater is applied to a ZDWS during the first year, flooded conditions are likely to occur, with a resultant negative effect on willow growth (Jackson and Attwood, 1996; Ministry of Environment and Energy, 2001). Thus the higher growth of the willows receiving wastewaters in the current study may not be replicated under flooded field conditions. This underlines the importance of the

increased yield of the inoculated willows compared to the non-inoculated willows when receiving rainwater, as this is the condition that is likely to exist under best practice installation guidelines. However, gradients in soil moisture and flooded conditions are known to affect mycorrhizal presence and colonisation in willows (Sumorok and Kiederzynska, 2007). ECM in particular are known to be negatively affected by flooding, while AMF are known to be somewhat more tolerant of flooded conditions (Lodge, 1989). Further examination of the effect of flooding on mycorrhizal colonisation is therefore warranted.

Recommendations

Poor maintenance of OWTS is one of the leading causes of failure of septic tank systems under the National Inspection Plan (EPA, 2015). A lack of engagement on the part of the OWTS owner has been identified as a contributing factor to the failure rate, with 67% of owners indicating that they did not seek any information on the operation and maintenance of their septic tank system (EPA, 2015). In addition, the owners of private drinking water wells have been found to be least aware of the threat from OWTS as a source of aquatic contamination, despite being the group at most at risk from poorly performing systems (Naughton and Hynds, 2014).

In light of these findings, the relatively high levels of maintenance required for the covered willow systems would be of concern. This maintenance includes annual coppicing, spraying of trees to combat infestations, herbicide treatment to control weeds, regular assessment of polythene and repair of any tears, and annual cleaning of the polythene with an anti-algal agent. A key aim of the National

Inspection Plan is to increase stakeholder engagement to promote best practice in the installation, operation and maintenance of OWTS (EPA, 2013). Taking the lead of the National Inspection Plan, any guidelines for the use of covered ZDWS in Ireland should incorporate clear instructions for maintenance of the system. Furthermore, annual inspection of the covered ZDWS should be included as a mandatory condition of the granting of planning permission for the construction of these systems.

Consideration should also be given to the performance of the [+ willow + cover] ZDWS during the summer months in the event of a prolonged period of non-occupancy of the household. The absence of wastewater input during this time period at the peak of the growing season, and hence peak transpiration demand, could lead to drought stress of the trees. A system to introduce wastewater/water during these time periods would need to be designed and trialled. For example, a system of roof rainwater harvesting which could be diverted to the wetland before the owners leave could be explored in future work. Such a rainwater harvesting unit would not necessarily increase the footprint of the willow system as rainwater could be directly channelled to the willow bed without the need for a holding reservoir. Obviously, in the case of the [- willow – cover] system, such water-shortage concerns would not apply.

For ZDWS in Denmark, an annual crop factor of 2.5 has been used in the system design (Ministry of Environment and Energy, 2003). In Ireland the only published willow crop factors are those of Curneen and Gill (2014, 2015), from studies in Dublin (east of Ireland) and Co. Wexford (south-east of Ireland). Most recently,

Curneen and Gill (2015) recorded an annual crop factor of 2.2, and a growing season (May – September) crop factor of 2.5, which agree very closely with the growing season crop factors of 2.0 - 3.6 calculated in this study (section 5.4.1). The willow crop factors recorded in the lysimeter study can be used to inform the crop factor values used for ZDWS used in the south of Ireland.

Future research

Areas of future research which arise from the current study include:

- Wastewater in this system was applied at the surface level of the soil. Traditionally in ZDWS, wastewater is discharged in the subsurface. Further studies should assess the impact on subsurface discharge on the evapotranspiration levels of the [+ willow + cover] and [- willow + cover] wetland types.
- Testing of willow-selected mycorrhizal inoculum under flooded conditions should be carried out. Flooded conditions are known to affect mycorrhizal presence on willow roots and waterlogging tolerance could be particularly relevant for use in a ZDWS scenario.
- It is not known how the movement of carbon to the mycorrhizal host is affected by coppicing cycles in willow SRC (Rooney *et al*., 2009). The occurrence of EMF can be affected by rotation periods of willow and poplar SRC, with some occurring more on three-year SRC rotations and others on six-year rotations (Hrynkiewicz *et al*., 2010). Exploration of mycorrhizal colonisation levels after successive harvest cycles of ZDWS is required to help explain the contribution from mycorrhiza over the life-time of a ZDWS.

- Additional selection over a wider range of mycorrhizal fungi, selection between particular isolates of, say, *G. mosseae* and *G. intraradices,* and evaluation of the selected inoculum over different willow species should also be trialled.

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Appendix 1 Constituents of DIEHARD™ Transplant

AMF	12.5 propagules per cubic centimetre: *Glomus mosseae* *G. intraradices* *G. claroideum* *G. etunicatum* *G. clarum* *G. deserticola* *G. microaggregatum*
ECM	180,400 colony forming units per cubic centimetre to include: *Pisolithus tinctorius* (155,400) *Rhizopogon occidentalis* (25,000).
Nitrogen fixing, phosphate solubilising, and growth promoting bacteria	1,000,000 colony forming units per cubic centimetre to include: Genus *Bacillus:* *B. megaterium* *B. subtilla* *B. amyloliquefaciens* *B. azotoformans* *B. licheniformis* *B. pumulus* *B. firmus* *B. coagulans* *Geobacillus stearothermophilus* *Paenibacillus polymyxa* Genus *Pseudomonas:* *P. putida,* *P. fluorescens* *P. aureofaceans* *P. durum* Genus *Streptomyces*: *S. lydicus* *S. griseus* *S. coelicolor*
Trichoderma	72,600 colony forming units per cubic centimetre. Genus *Trichoderma:* *Trichoderma hamatum* *T. harzianum* *T. viride* *T. reesei*
Humic Acid	21 % derived from *Leonardite*
Seaweed Extract	10 % *Ascophylum nodosum*
Yucca Plant Extract	4 % *Yucca schidigera*
Root Promoting Vitamins	B, B2, B3, B5, B6, B7, B9, B12, C, K, Biotin
Amino Acids	Animal and plant proteins
Additional organic chemicals	Purines, Pyrimidines, Fulvic Acid
Horta-Sorb® MD Water Gel **Particle Size** **Percent Soluble** **Absorption Rate**	Minimum 29 % cross linked copolymer 0.8 mm to 2.0 mm, maximum 5 % < 1.0 mm Less than 5 % 300 - 400 times in distilled water

Appendix 2 Research outputs and training

Conference presentations – academic

Zero-discharge willow wetland for on-site domestic wastewater treatment in Ireland. Environ 2013, NUIG.

Endomycorrhiza-ectomycorrhiza inoculum application to a zero-discharge willow wastewater treatment system. Irish Plant Scientists' Annual Meeting 2014, UCC.

The application of a commercial mycorrhizal fungal inoculum in a willow wastewater treatment system. Environ 2014, TCD.

Towards the development of a novel zero-discharge willow wastewater treatment system. Environ 2016, UL

Awards for PhD research

Winner - UCC Science For All - April 2013

Winner – UCC Doctoral Showcase - June 2013

Winner – *Irish Independent*/Higher Education Authority "Making an Impact" - March 2012

PhD Education and Training

PG 6014 Scientific Outreach and Communication – May 2014, UCC

ST 6013 Statistics and Data Analysis for Postgraduate Research Students – August 2013, UCC

MS Excel and MS Word Level 2 – March 2013, UCC

ArcGIS for Postgraduate Students - May 2013, UCC

Symposium on zero-discharge willow wetland systems - June 2012, Wexford

Erasmus Intensive Programme: River Restoration in the Atlantic Arc – April 2012, University of Oviedo

Carr Communications training – March 2012, UCC

PG 6009 Graduate Information Literacy Skills – Feb 2012, UCC

EPA/TCD International Symposium on Wastewater Treatment and Disposal Systems - September 2012, TCD

Hugh Kearns Turbocharge Your Writing for year 3 students – February 2012, UCC

Creativity in the context of research with Kevin Byron – November 2011, UCC

Irish Conference on Constructed Wetlands - October 2010, UCD

PG 6001 Scientific Training for Enhanced Postgraduate Study – May 2010, UCC

PG 6003 Teaching and Learning for Graduate Students - Nov 2009 – May 2010, UCC

Turbocharge your Writing – October 2009, UCC

Teaching

Agri-Food Graduate Development Programme - May 2012 – present, UCC and UCD

EV 3010 Freshwater Resources Management - April 2015, UCC

MSc Organic Horticulture – May 2012, UCC

Other Science and Research Communication Awards

Winner – FameLab International, June 2013

Winner – FameLab Ireland, April 2013

Shortlisted *Guardian*/Wellcome Trust Science Writing Prize, September 2013

Science Communication on TV and radio

Researcher, writer and presenter: The Science Squad Series 2 and 3, July 2013 – September 2014.

Researcher, writer and presenter: 10 Things to Know About…, June 2015 – present

Researcher and panellist on Newstalk Futureproof, June 2015 - present

Invited talks, keynotes and presentations

Forum Wissenschaft im Dialog. November 2015, Nuremburg.

Irish Science Teachers' Association Annual Meeting. April 2014, NUIG.

Occupational Hygienists' Association of Ireland Annual Meeting. February 2014, Portlaoise.

Pirelli "Spinning the Wheel". January 2014, Milan.

UCD School of Biological and Environmental Science Seminar Day – December 2013.

Undergraduate Awards Debate. November 2013, UCD.

Echoes of FameLab. November 2013, Prague.

UCC Alumni keynotes: September-November 2013, London, Chicago, Boston, New York.

TEDx. September 2013, Dublin.

European Researchers' Night. September 2013, Natural History Museum, London.